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Neoplastic Transformation: Implications for the Etiology
of Human Breast Cancer

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INTRODUCTION

Recent work in our laboratory has established the importance of a c-Src-dependent phosphorylation site on the EGFR, namely, Tyr 845. Mutagenesis studies have demonstrated that phosphorylation of this site, which resides in the catalytic portion of the EGFR molecule, plays a crucial role in EGF-induced mitogenesis. My current research has demonstrated that the EGFR becomes phosphorylated in response to a variety of extracellular mitogens including cytokines (growth hormone), steroids (estrogen), and G-protein coupled ligands (lysophosphatidic acid and endothelin). These alternative agonists induce phosphorylation of EGFR Tyr 845 and trigger the co-association between the EGFR and c-Src. Moreover, introduction of a Y845F mutant form of the EGFR into fibroblasts or MCF7 breast tumor cells decreases DNA synthesis in response to all the alternative agonists listed above, indicating that phosphorylation of Tyr 845 plays a crucial role in cellular proliferation triggered by a variety of extracellular stimuli. These findings suggest that the phosphorylation of the EGFR may be a common signalling event shared by several pathways within the cell. The mitogenic signalling pathways downstream of EGFR Tyr 845 phosphorylation remain to be clearly elucidated, although recent evidence implicates one of the STATs (signal transducers and activators of transcription), classically described as a cytokine receptor effector, in modulating EGF-induced mitogenesis.

ANNUAL SUMMARY

Increased expression or activity of tyrosine kinases, and the subsequent deregulation of downstream signalling cascades, is thought to play a major role in the genesis and progression of many different human cancers. The nonreceptor tyrosine kinase c-Src, and the epidermal growth factor receptor (EGFR), are especially implicated in breast cancer (reviewed in Biscardi et al., 1999, 2000). Overexpression of the human EGFR (HER1) and/or the related family member HER2 occurs in 20-30 % of human breast tumors and, in the case of HER1, correlates with the loss of estrogen responsiveness (Sainsbury et al., 1985; Singletary et al., 1987; Koenders et al., 1991; Slamon et al., 1986). Further work suggests that HER1 may be involved in later, more aggressive stages of breast cancer (Sainsbury et al., 1987; Toi et al., 1991; Battaglia et al., 1988). c-Src also plays an important, but as yet undefined, role in human breast cancer. Ottenhalf-Kalff et al. (1992) showed that 100% of primary human breast tumors examined displayed increased tyrosine kinase activity, and 70% of this cytosolic activity could be attributed to c-Src.

The dual overexpression of both the EGFR and c-Src in many of the same human tumor types suggests that they may interact in some manner to control cellular events which could lead to transformation and tumorigenesis. Indeed, our laboratory has used fibroblast model systems, human breast cancer cell lines, and tumor tissues to demonstrate that overexpression of both these kinases results in their physical association, increased tumorigenesis in nude mice, and enhanced signal transduction emanating downstream of the receptor (Maa et al., 1995; Biscardi et al., 1998).

Since our preliminary data in the fibroblasts indicated that a causal role for c-Src and the EGFR did exist, the first aim of my research was to see if similar conclusions about c-Src and the EGFR could be drawn from human breast tumor samples and cell lines. To this end, I obtained several breast tumor tissue samples from the Tissue Procurement Facility at the University of Virginia, as well as several breast cancer cell lines that expressed varying levels of EGFR and/or c-Src. These studies resulted in a publication in the journal *Molecular Carcinogenesis* (Biscardi et al., 1998; reprint appended to this Final Report). Briefly, we found that the results obtained from the mouse fibroblast system did indeed apply to human breast cancer: overexpression of both c-Src and the EGFR correlated with increased activation of the EGFR effector MAPK and increased tumorigenicity. In addition, in breast tumor samples or cell lines where both kinases are overexpressed, the EGFR physically associates with c-Src and becomes phosphorylated on two novel tyrosyl residues, as was the case in the fibroblast model system. These findings suggest that the unregulated interaction between c-Src and the EGFR may result in increased transforming potential in breast cells.

The next object of my research was to identify and characterize the two novel phosphorylations observed on the c-Src-associated EGFR. These results were published in the *Journal of Biological Chemistry* (Biscardi et al., 1999; see appended reprint) and in the *Proceedings of the National Academy of Sciences* (Tice et al., 1999). Phosphotryptic peptide mapping revealed that these phosphorylations occurred on Tyr 845 and Tyr 1101. Tyr 845 lies within the catalytic domain, in a highly conserved, critical region of the

tyrosine kinase activation lip. Mutation of the analogous residue in numerous other tyrosine kinases ablates tyrosine kinase activity (Ellis et al., 1986; Fantl et al., 1989; van der Geer and Hunter, 1991; Vigna et al., 1994; Mohammadi et al., 1996; Zhang et al., 1998). The critical nature of this phosphorylation in other tyrosine kinases suggested that Tyr 845 phosphorylation might play an important role in EGFR function as well. In this regard, Tyr 845 phosphorylation is observable only in cells where both c-Src and the EGFR are overexpressed and are physically associated, a situation that is often found in breast cancer cells and that roughly correlates with increased tumorigenicity (Biscardi et al., 1998). This finding provides compelling evidence for studying how phosphorylation of this residue contributes to tumorigenesis.

To this end, a mutant form of the EGFR expressing a tyrosine to phenylalanine mutation at position 845 was introduced into either COS cells or 10T1/2 mouse fibroblasts, and the resulting effect on EGFR kinase activity determined. Interestingly, while mutation of the analogous tyrosine in other tyrosine kinase molecules ablates catalytic function, mutation of Tyr 845 in the EGFR did not affect its kinase activity (Tice et al., 1999). However, cells expressing the Y845F mutant EGFR were severely impaired in their ability to respond mitogenically to EGF, indicating that phosphorylation of Tyr 845 is crucial for cellular proliferation (Biscardi et al., 1999; Tice et al., 1999).

Interestingly, introduction of the Y845F mutant EGFR into fibroblasts also decreased proliferation in response to serum and to lysophosphatidic acid (LPA), the chief mitogenic component of serum (Tice et al., 1999). This finding suggested that the EGFR might be interacting with G-protein mediated signaling pathways in the cell. Previous evidence from other labs demonstrates the existence of cross-talk between the EGFR and G-protein coupled receptors (GPCRs) or cytokine receptors. LPA, which binds a Gi/Gq linked receptor, has been shown to induce phosphorylation of the EGFR and the subsequent activation of downstream signalling molecules (Daub et al., 1997; Luttrell et al., 1997). Studies using kinase inactive c-Src, pharmacological inhibitors of c-Src, or expression of CSK (Cellular Src Kinase), which inactivates c-Src, have shown that c-Src kinase activity is required for the LPA- dependent phosphorylation of the EGFR (Daub et al., 1997; Luttrell et al., 1997).

Thrombin (THR), and endothelin (ET), other GPCR agonists, also induce the phosphorylation of the EGFR in when the required G protein is expressed in COS cells (Daub et al., 1996, 1997). In addition, Bokemeyer et al. (2000) have shown that angiotensin, which binds a GPCR, also triggers EGFR phosphorylation in a c-Src dependent manner; and furthermore, kinase activity of the EGFR is required for angiotensin-induced DNA synthesis. Additionally, EGFR phosphorylation can be triggered by growth hormone (GH), a cytokine receptor coupled agonist, in mouse liver (Yamauchi et al., 1997); and by other external stimuli including UV light, cellular stress, and activation of integrins (reviewed in Zwick et al., 1999; Harris, 2000). The multiplicity of pathways impinging on EGFR phosphorylation and activation suggests that rather as existing as a separate signaling module which responds only to its classically defined ligands, EGF or TGF α , the EGFR may instead act as a central regulator for a variety of signalling events within the cell. In this regard, my current work has thus focussed on elucidating the contribution of EGFR Tyr 845 to signalling downstream of G protein- and cytokine- receptor and steroid hormone- coupled agonists.

The finding that serum and LPA-induced DNA synthesis was blocked by introduction of the Y845F mutant EGFR was both unexpected and intriguing, and I chose to investigate this phenomenon in further detail. I first established that Tyr 845 became phosphorylated as a result of activation of G protein or cytokine receptor coupled pathways in the 10T1/2 fibroblast model system. Fibroblasts expressing either wild type EGFR and wild type c-Src (wtR/K+ src cells) or wild type EGFR and kinase inactive c-Src (wtR/K- src cells) were stimulated for 10 minutes with EGF, ET, GH, isoproterenol (ISO), or LPA. Cells were lysed in RIPA buffer and subjected to an EGFR immunoprecipitation followed by a phosphotyrosine immunoblot (Fig. 1, Appendix). While EGF caused the greatest degree of EGFR tyrosine phosphorylation, significant increases in EGFR phosphorylation were also elicited by ET, GH, and LPA; and by THR (data not shown). ET and GH elicited the more robust responses, while isoproterenol induced little increase in EGFR phosphorylation, suggesting that the Gs α coupled pathway and the EGFR pathway do not interact. These agonists were able to induce tyrosyl phosphorylation of the EGFR in both wtR/K+src and wtR/K-src cell lines. However, the level of phosphorylation was lower in the wtR/K-src cells, indicating that c-Src activity is necessary for these phosphorylations to occur, as has been previously shown in the case of LPA (Daub et al., 1997; Luttrell et al., 1997). These results demonstrate that the EGFR becomes phosphorylated in fibroblasts in response to a variety of extracellular stimuli, and because of this, the EGFR may participate in a large array of signalling pathways within the cell.

To determine if the EGFR and c-Src were able to physically associate in response to these agonists, as was previously shown to occur in response to EGF (Maa et al., 1995; Biscardi et al., 1998, 1999), c-Src immunoprecipitates from CHAPS lysates of wtR/K+src cells treated for 10 minutes with the above agonists were subjected to an immune complex kinase reaction (Fig. 2). Treatment of the cells with EGF, ET, GH, LPA and THR induced the physical association between c-Src and EGFR. LPA treatment resulted in a very robust association, while ET and GH (Fig. 2), and THR (data not shown) triggered association to a lesser extent. Treatment with these agonists did not appear to affect the kinase activity of c-Src, as measured by autophosphorylation. These results are consistent with those of Lefkowitz and colleagues (1997), who demonstrated that LPA is capable of triggering the formation of a c-Src/EGFR heterocomplex in COS cells. However, it remains unclear whether the GPCR and cytokine-receptor coupled agonists act to increase EGFR phosphorylation by increasing the EGFR's ability to bind to the receptor, or to autophosphorylate; or by increasing the ability of c-Src to phosphorylate the receptor. While c-Src kinase activity does not appear to be altered by treatment with these agonists, it is possible that treatment with GH, LPA, ET and THR results in the relocalization of c-Src to a particular subcellular signalling complex, where it is in a more optimal position to phosphorylate the EGFR.

To gain further insight into the GPCR-and cytokine coupled receptor mediated phosphorylation of the EGFR, tryptic phosphopeptide mapping was performed on the c-Src associated EGFR derived from wtR/K+src cells and wtR/K-src cells treated with the panel of agonists. GH, LPA and ET all triggered the phosphorylation of Tyr 845 (previously identified in response to EGF (Maa et al., 1995; Biscardi et al., 1999)) in the c-Src associated EGFR derived from wtR/K+ cells (Fig. 3, top row). Interestingly, no phosphorylation of Tyr 845 was observed in response to any of the treatments in wtR/K-

cells (Fig. 3, bottom row), indicating that the phosphorylation of Tyr 845 requires c-Src kinase activity. The other c-Src dependent phosphorylation, Tyr 1101, was also present in these cells, but agonist treatment did not increase its phosphorylation over unstimulated levels. Additionally, the tryptic map derived from c-Src associated EGFR from ISO treated 5HR cells showed a barely visible pattern of spots, with no Tyr 845 phosphorylation observed (Fig. 3, inset). Thus, unlike the Gq (ET) and Gi/Gq (LPA) coupled pathways, the Gs α coupled pathway is unlikely to interact with EGF-mediated signalling. These results suggest that the phosphorylation of EGFR Tyr 845 is a more general phenomenon which occurs in response to the activation of a number of signalling pathways, and that c-Src kinase activity is required for its phosphorylation regardless of the signalling mechanism involved.

The fact that Tyr 845 resides in the activation loop of the kinase domain indicates that phosphorylation of this residue may in some way affect signalling through the EGFR. Given the fact that other GPCR and cytokine receptor mediated pathways trigger phosphorylation of EGFR Tyr 845, it is possible that this phosphorylation site may be necessary for mitogenesis in response to these other agonists. To test this hypothesis, 10T1/2 fibroblasts overexpressing wild-type c-Src (K+ cells) were transiently transfected with either wild type or Y845F EGFR. Following a 24 hour post-transfection recovery period, cells were serum starved for 30 hrs, then incubated with bromodeoxyuridine (BrdU) and one of the panel of agonists for 19 hours. Cells were then processed for double immunofluorescence labelling as described previously (Biscardi et al., 1999; Tice et al., 1999). Fig. 4 shows that expression of a Y845F mutant EGFR resulted in decreased DNA synthesis in response to EGF, as well as to the GPCR ligands LPA and ET, and to the cytokine receptor coupled ligand, growth hormone (GH). Another GPCR ligand, thrombin, did not act as a mitogen for these cells. Mitogenesis in response to platelet derived growth factor (PDGF), which activates the PDGF receptor tyrosine kinase, was not affected by expression of the Y845F mutant EGFR, which indicates that this mutation does not act as a nonspecific inhibitor of all mitogenic signalling in the cell. Mitogenesis in response to another tyrosine kinase receptor ligand, insulin-like growth factor I, was only partially inhibited by expression of the mutant EGFR. The fact that LPA-, ET-, and GH- induced mitogenesis is dependent on the ability to phosphorylate EGFR Tyr 845 suggests that this phosphorylation site is a common, critical target downstream of a wide variety of mitogenic stimuli.

In addition to being a mediator of signalling resulting from GPCRs and the growth hormone receptor, there is increasing evidence that crosstalk exists between EGF-mediated and estrogen-mediated pathways. It has long been known that EGF is able to induce transcription of estrogen-responsive genes via activation of the AF-1 (ligand-independent) region of the estrogen receptor (Ignar-Trowbridge et al., 1992, 1993). It has been hypothesized that the EGFR triggers activation of the mitogen-activated protein kinase (MAPK), which phosphorylates the estrogen receptor at position 118 in the AF-1 region (Kato et al, 1995; Bunone et al., 1996). More recent work shows that c-Src signalling can be regulated by estrogens. For example, estrogen treatment causes rapid (within 5 minutes) increases in c-Src autophosphorylation and kinase activity in MCF7 breast cancer cells, and the concomitant activation of the MAPK pathway (Migliaccio et al., 1993, 1996, 1998). Moreover, both c-Src kinase activity and MAPK activity are necessary for estrogen-induced proliferation of MCF7 cells; and the mitogenic effects of

estrogen can be divorced from its transcriptional activity (Castoria et al., 1999). An intriguing hypothesis for the crosstalk between estrogen and c-Src kinase pathways could involve the c-Src mediated phosphorylation of EGFR Tyr 845. Since Tyr 845 phosphorylation appears to be a general response to activation of EGF, cytokine, and G protein mediated pathways, I decided to see how estradiol treatment might affect EGFR signalling.

MCF7 human breast cancer cells were cultured in phenol-red free media and charcoal stripped serum 24 hr prior to transfection. Cells were then transiently transfected with wild-type EGFR, stimulated with either 10 ng/ml EGF or 10 μ M estradiol for 5 min, and the EGFR was immunoprecipitated and examined for levels of tyrosyl phosphorylation. The preliminary result shown in Fig. 5 suggests that estradiol is indeed capable of inducing EGFR phosphorylation. Further experiments to verify this result are currently underway. I am also investigating whether estradiol is directly capable of inducing phosphorylation of the EGFR Tyr 845. Unfortunately, it has been difficult to express high amounts of the receptor in MCF7 cells to result in a sufficient number of counts for phosphotryptic peptide mapping. These studies are still ongoing.

Since phosphorylation of EGFR Tyr 845 was critical for GPCR and cytokine receptor ligands, I next examined the possibility that estradiol-induced DNA synthesis could be decreased by introduction of the Y845F mutant EGFR into human breast cancer cells. To answer this question, MCF7 breast cancer cells cultured as described above were transiently transfected with either wild type or Y845F EGFR, allowed to recover for 24 hr, serum starved for 30 hours in phenol red free-media, then incubated for 19 hours with BrdU and either EGF (40 ng/ml) or estradiol (3 μ M). Cells were then processed for double immunofluorescence labelling as described (Tice et al., 1999). MCF7 cells responded mitotically to both EGF and estradiol (Fig. 6). Moreover, expression of Y845F mutant EGFR resulted in a striking decrease of both EGF- and estradiol- induced DNA synthesis. In fact, expression of the Y845F mutant decreased DNA synthesis to unstimulated levels, as was seen in response to the other agonists in 5H cells. Thus, phosphorylation of EGFR Tyr 845 is a critical event for estrogen induced mitogenesis. Taken together with the above results, the c-Src mediated phosphorylation of Tyr 845 appears to be a central, critical event that is crucial for mitogenesis downstream of various G- protein coupled, cytokine, and estrogen receptors.

In addition to studying the role of Tyr 845 phosphorylation in EGFR crosstalk, our laboratory has also begun to elucidate which biochemical pathway(s) downstream of Tyr 845 phosphorylation lead to mitogenesis. Initial studies comparing wtR/K+src cells and wtR/K-src cells (in which EGFR Tyr 845 phosphorylation was not observed by tryptic mapping) did not reveal any differences in the EGF- dependent activation/phosphorylation of several signalling molecules downstream of the EGFR including Shc, phospholipase C γ , MAPK, STAT3, and PI3-kinase (DA Tice and JS Biscardi, unpublished results). Moreover, COS cells which were transiently transfected with either wild type or Y845F mutant EGFR likewise did not show any differences in EGFR autophosphorylation activity, Shc phosphorylation, or MAPK activation (Tice et al., 1999). In similar studies, MCF7 breast cancer cells were transiently transfected with either the wild type or mutant EGFR, and the ability of the agonist panel to trigger MAPK activation was determined. Again, no difference in MAPK activation was observed between wild type and Y845F mutant EGFR in this cell line. These negative

results suggest that an as yet undescribed pathway controls growth downstream of Tyr 845 phosphorylation.

Further work from our laboratory and that of our collaborator, Dr. Corinne Silva, points to a role for STAT5b in regulating signalling downstream of EGFR Tyr 845 phosphorylation, in that decreased EGF-dependent STAT5b phosphorylation is observed in wtR/K- cells with respect to wtR/K+ cells (Kloth et al., submitted). To see if Y845F EGFR directly affected the ability of EGF to trigger STAT5b phosphorylation, and if this STAT could be activated in response to the alternative agonists, K+ fibroblasts were transiently transfected with tagged STAT5b and either wild type or mutant EGFR, treated for 10 min with the various agonists, and the levels of STAT5b phosphorylation determined. Fig. 7 shows that EGF-induced STAT5b tyrosine phosphorylation levels are reduced in cells expressing the Y845F mutant EGFR when compared to cells expressing the wild type receptor. However, GH-induced STAT5b phosphorylation is unaffected by the Y845F mutation. The other GPCR agonists did not affect STAT5b phosphorylation, as was expected.

Interestingly, studies using an antibody directed against phosphorylated Tyr 699 of STAT5, the well-described C-terminal STAT tyrosyl phosphorylation, was not affected by expression of the mutant EGFR, suggesting that another tyrosine is phosphorylated downstream of EGFR Tyr 845 phosphorylation (data not shown). Indeed, Olayioye et al. (2000) have shown that there is an additional c-Src dependent tyrosine phosphorylation site on STAT5. Kloth et al. (submitted) also show that STAT5b phosphorylation and downstream transcriptional activity are dependent on c-Src kinase activity.

In further studies, I investigated whether STAT5b signalling was necessary for EGF- or GH- induced mitogenesis. K+ fibroblasts were transiently transfected with either hemagglutinin-tagged wild-type STAT5b or a dominant negative form of STAT5b that is impaired in its ability to mediate transcriptional activation of STAT responsive genes (C. Silva, personal communication). Cells were then processed for double immunofluorescence staining as described above, and the EGF- or GH- induced incorporation of BrdU into newly synthesized DNA was measured. Fig. 8 shows that while expression of wild type STAT5b did not affect BrdU incorporation in these cells, expression of the dominant negative STAT5b ablated both GH- and EGF- induced DNA synthesis to basal levels.

The last aspect of my work this year dealt with elucidating the function of the other c-Src-dependent phosphorylation of EGFR, namely, Tyr 1101; and determining the region of the EGFR where c-Src binds. Tyr 1101 is present in the carboxy terminus of the EGFR, and thus may serve as a docking site for EGFR substrate molecules. In vitro evidence has shown that c-Src can bind a peptide containing a phosphorylated Tyr 1101 via a PTyr/SH2 interaction (Lombardo et al., 1995). Thus, I speculated that upon EGF stimulation, c-Src creates its own binding site on Tyr 1101 of the EGFR. To this end, a Y1101F mutant form of the EGFR was created.

To see if the Y1101 site was involved in binding to c-Src, COS cells were transfected with c-Src and either wild-type or Y1101F mutant EGFR cDNA, stimulated with EGF, lysed, and subjected to c-Src immunoprecipitation. Both forms of the EGFR were able to co-precipitate with c-Src, indicating that the Y1101 site does not contribute to c-Src binding (data not shown). I am currently studying whether cells expressing a

mutant Y1101F EGFR are impaired in their ability to synthesize DNA in response to EGF and the alternative agonists.

I next investigated the hypothesis that c-Src binds to another site in the EGFR c-terminus via a P_{tyr}/SH2 interaction. To this end, NIH3T3 fibroblasts that express the EGFR bearing individual point mutations at Tyr 992, 1068, 1086, 1148, 1173, or all 5 of these sites (F5), were transiently transfected with wild-type c-Src, and c-Src co-immunoprecipitation experiments were carried out as performed above. c-Src was found to bind to all mutant forms of the EGFR, although binding to the F5 mutant was somewhat decreased. This finding suggests that c-Src does not have a specific binding site on the receptor; and in the absence of a preferred site c-Src may bind to alternative sites. Indeed, other EGFR substrates demonstrate are able to bind multiple sites on the receptor C-terminus in this manner.

Key Research Findings:

- Phosphorylation of the EGFR can be triggered by agonists besides EGF: namely, those that work through G protein coupled receptors (LPA, ET, THR) and cytokine receptors (GH).
- These agonists are also capable of inducing association between c-Src and the EGFR.
- Tyr 845, a critical residue in the activation lip of the EGFR catalytic domain, becomes phosphorylated in response to a number of agonists including EGF, ET, LPA, and GH.
- C-Src kinase activity is required for the phosphorylation of Tyr 845 to occur downstream of the above stimuli.
- Mutation of Tyr 845 to phenylalanine results in ablation of EGF, LPA, ET, GH and estrogen dependent mitogenesis.
- STAT5b tyrosyl phosphorylation is decreased in cells expressing Y845F EGFR.
- The transcriptional activity of STAT5b is required for both GH- and EGF- induced DNA synthesis.

Reportable Outcomes:

Manuscripts.

Biscardi JS, Belsches AP, Parsons SJ (1998). Characterization of human epidermal growth factor receptor and c-Src in human breast tumor cells. *Mol. Carcinogen.* 21: 261-272.

Tice DA, Biscardi JS, Nickles AL, and Parsons SJ (1999). Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proc. Natl. Acad. Sci USA* 96: 1415-1420.

Biscardi JS, Maa M-C, Tice DA, Cox ME, Leu T-Z, and Parsons SJ (1999). c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr 845 and Tyr 1101 is associated with modulation of receptor function. *J. Biol. Chem.* 274: 8335-8343.

Biscardi JS, Tice DA, Parsons SJ (1999). C-Src, receptor tyrosine kinases, and cancer (1999). *Adv. Cancer Res.* 76: 61-119.

Biscardi JS, Ishizawar RC, Silva CM, Parsons SJ (2000). Epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast Cancer Res.* 2: 203-210.

Belsches-Jablonski AP, Biscardi JS, Peavy DR, Tice DA, Romney DA, and Parsons, SJ (2000). Src family kinases and heregulin cooperate to promote human breast cancer cell growth and survival. Submitted.

Kloth MT, Biscardi JS, Tice DA, Parsons SJ, and Silva CM (2000). STAT5b is a mediator of synergism between c-Src and the EGF receptor in two models of tumorigenesis. Submitted.

Abstracts.

Biscardi JS, Tice DA, Parsons SJ. Crosstalk between G protein coupled receptor and EGF-dependent pathways: Implications for c-Src mediated phosphorylation of EGFR Tyr 845. Gordon Research Conferences, Peptide Growth Factors, 1998.

Biscardi JS, Tice DA, Parsons SJ. c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr 845 and Tyr 1101 is associated with modulation of receptor function. Seminar of Cancer Researchers in Virginia, American Cancer Society, 1999.

Biscardi JS, Tice DA, Sproles D, Parsons SJ. The c-Src mediated phosphorylation of epidermal growth factor receptor tyrosine 845 is a critical event in multiple cellular signalling pathways. Era of Hope DOD Breast Cancer Research Program Meeting, 2000.

CONCLUSIONS:

My work has shown that the synergy observed between c-Src and the EGFR in a fibroblast model system also occurs in human breast cancer. Overexpression of c-Src and the EGFR results in the physical association of these two molecules, the c-Src dependent phosphorylation of the receptor of tyrosines 845 and 1101, and increases in cellular proliferation and tumorigenicity. Tyrosine 845 phosphorylation is dependent on c-Src kinase activity, suggesting that it is directly phosphorylated by c-Src. Mutation of this tyrosine to a phenylalanine reduces EGF-induced cellular proliferation to basal, nonstimulated levels.

In addition to EGF, multiple extracellular mitogens are capable of triggering EGFR phosphorylation and association with c-Src. These include the GPCR ligands ET and LPA; and the cytokine receptor ligand GH. Moreover, these alternative mitogens also cause phosphorylation of EGFR Tyr 845, again in a c-Src dependent manner. Mutation of EGFR Tyr 845 causes a loss of mitogenic signalling in response to these alternative mitogens in fibroblasts, as well as to the steroid hormone estrogen in breast cancer cells. These findings suggest that phosphorylation of the EGFR at Tyr 845 acts as a central mediator in a wide variety of mitogenic signalling pathways.

Which downstream effector(s) become activated in response to the Tyr 845 phosphorylation triggered by these agonists remains an open question. The most well-described EGFR pathway involving activation of SHC and MAPK is not affected by introduction of the Y845F mutant EGFR. However, our recent data show that the cytokine effector STAT5b is involved in propagating the EGF-induced signal from the EGFR. Introduction of the Y845F mutant EGFR decreases the amount of STAT5b phosphorylation in response to EGF treatment, but not in response to GH or the other GPCR agonists. Introduction of a dominant negative form of STAT5b decreases both EGF- and GH-induced DNA synthesis to unstimulated levels, demonstrating the importance of STAT5b to these mitogenic signalling pathways.

It is still unclear what EGFR effectors are involved in signaling from LPA, ET, GH, and estrogen. Recent work by Fukuhara et al. (1999, 2000) shows that activation of Gq and G12/13 linked receptors signals downstream to ERK5/Big MAPK in a Ras-independent manner. I have obtained a sample of ERK5/BMK cDNA and plan to perform transfections along with wild type or Y845F EGFR to see if this pathway is involved. Other work in our laboratory centers around the identification of proteins that bind to phosphorylated Tyr 845, in an effort to elucidate new signalling pathways.

The phosphorylation of EGFR Tyr 845 appears to be a very tightly regulated event (Biscardi et al., 1999). Our fibroblast and breast tumor cell model systems show that increased expression of c-Src and the EGFR may lead to aberrant Tyr 845 phosphorylation, which in turn triggers increased cellular proliferation and tumorigenesis. In this regard, Tyr 845 phosphorylation is not observed in normal cells or in cells where c-Src and the EGFR are expressed at basal levels. This finding makes Tyr 845 a compelling target for clinical intervention, in that therapies directed against Tyr 845 would likely target only the tumor cells where this site is phosphorylated.

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Treatment of fibroblast cells with multiple agonists results in phosphorylation of the EGFR

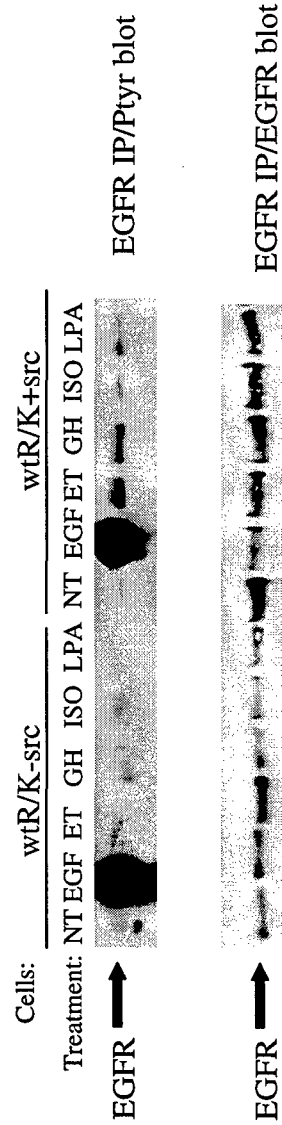
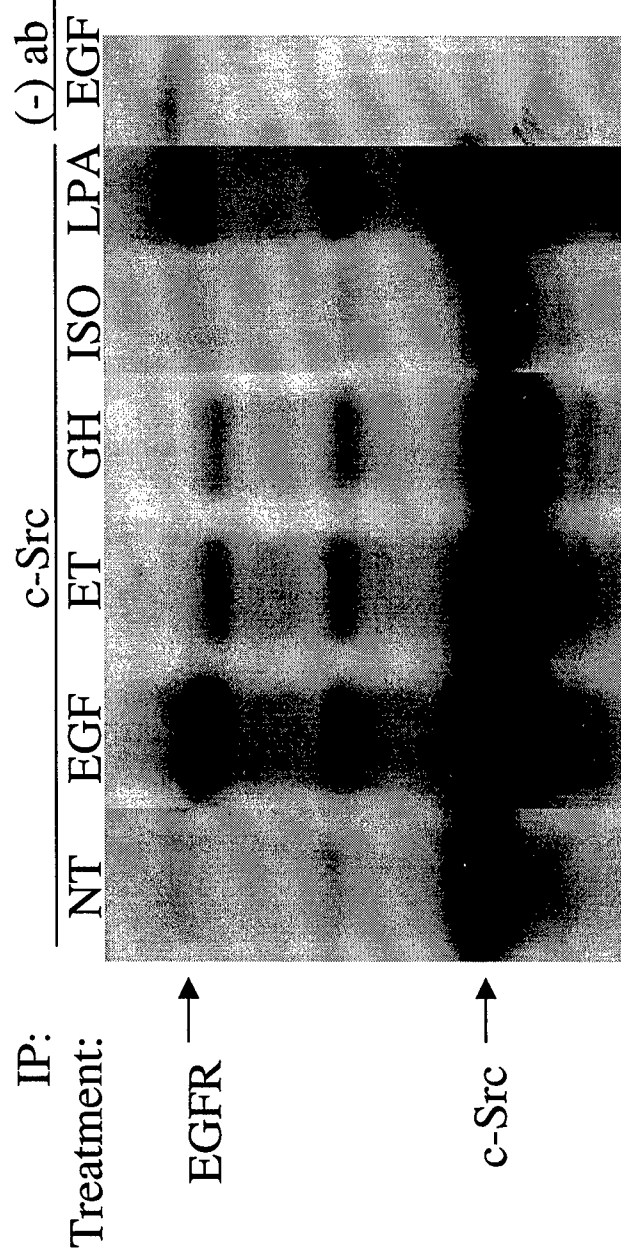


Fig.1. wtR/K+src or wtR/K-src cells were treated for 10 min with EGF (100ng/ml), endothelin (ET; 10 μ M), growth hormone (GH; 500 ng/ml), isoproterenol (ISO; 10 μ M) or lysophosphatidic acid (LPA; 10 μ M), lysed in RIPA buffer, and subjected to EGFR immunoprecipitations using Mab F4. Phosphorylated EGFR was visualized by immunoblotting with Ptyr antibody RC20 and ECL (top panel), while overall EGFR was visualized by immunoblotting with EGFR Mab F4 (bottom panel). The blot shown is representative of 5 independent experiments.

Multiple Agonists Induce EGFR Association with c-Src



c-Src IP/kinase
K+Src/wtR
cells

Fig. 2. wtR/K+src cells were treated with the various agonists for 10 min, lysed in CHAPS, and c-Src immunoprecipitated with Mab GD11. C-Src immunoprecipitates were subjected to an in vitro kinase reaction using $MnCl_2$ and γ ^{32}P -ATP. Phosphorylated proteins co-precipitating with c-Src were separated on SDS-PAGE and visualized by autoradiography.

Endothelin, growth hormone, LPA, and EGF all induce phosphorylation of Tyr 845 of the c-Src associated EGFR, and this phosphorylation is dependent on c-Src kinase activity.

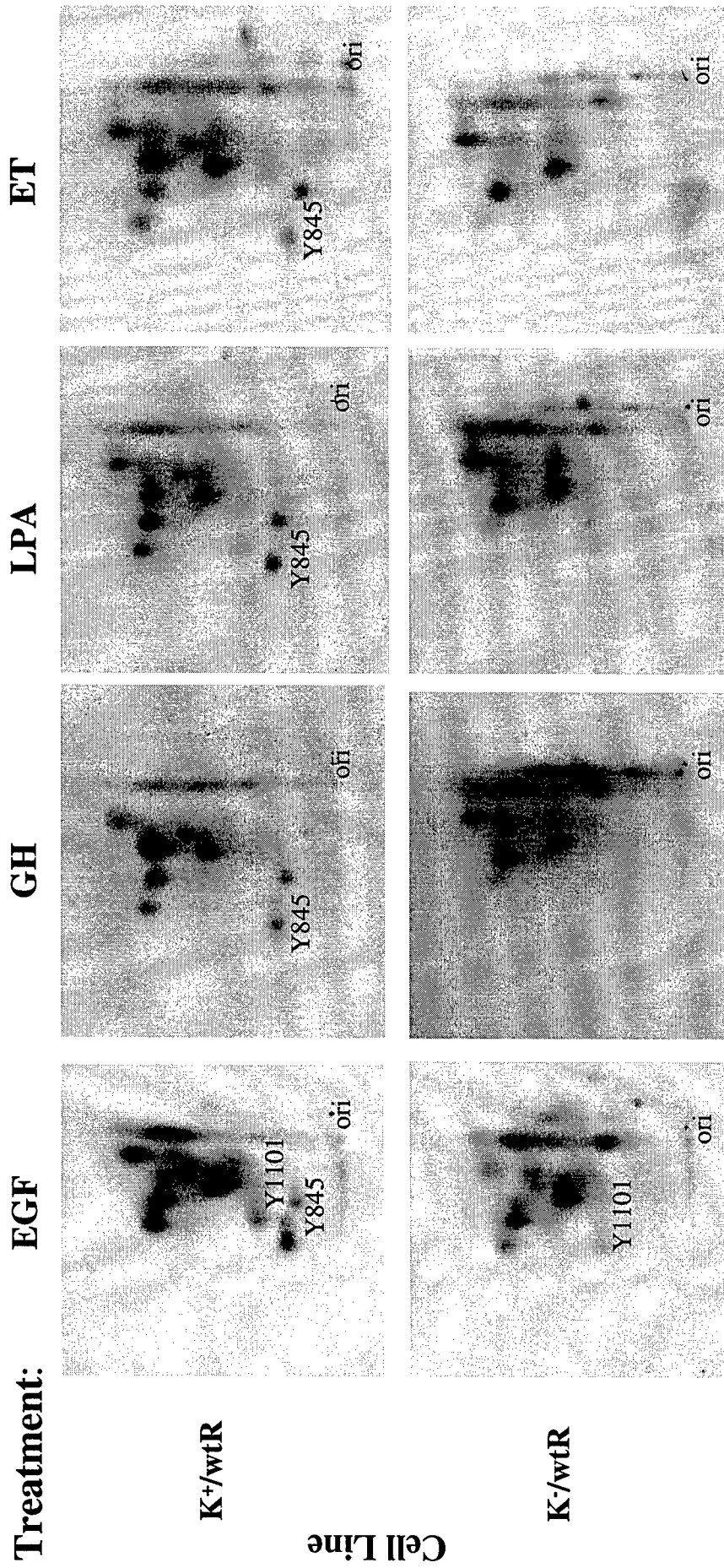


Fig.3. K+Src/wtR or K-Src/wtR cells were stimulated for 10 min with the indicated agonist, and then were processed for in vitro c-Src immunocomplex kinase reactions. The c-Src associated EGFR was excised and digested with trypsin. The maps shown are representative of 3 independent experiments.

K+/wtR, isoproterenol treated



Y845F Mutant EGFR Decreases DNA Synthesis in Response to a Variety of Extracellular Stimuli

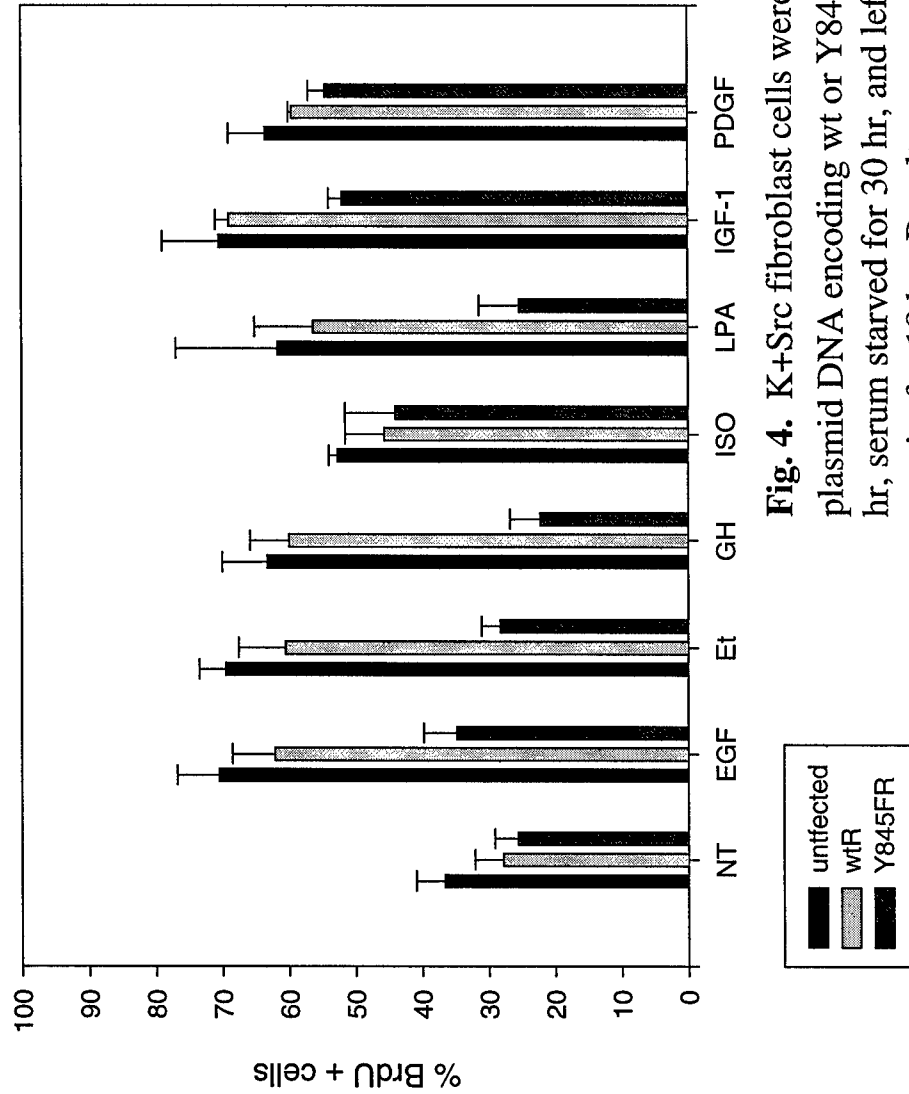


Fig. 4. K+Src fibroblast cells were transfected with plasmid DNA encoding wt or Y845F EGFR, cultured for 24 hr, serum starved for 30 hr, and left untreated or treated with agonist for 18 hr. Results are expressed as the mean percent +/- SEM of cells expressing the EGFR that were positive for BrdU incorporation. 35-50 cells were analyzed for each variable in at least 3 independent experiments.

Estradiol treatment of MCF7 breast cancer cells induces phosphorylation of the EGFR

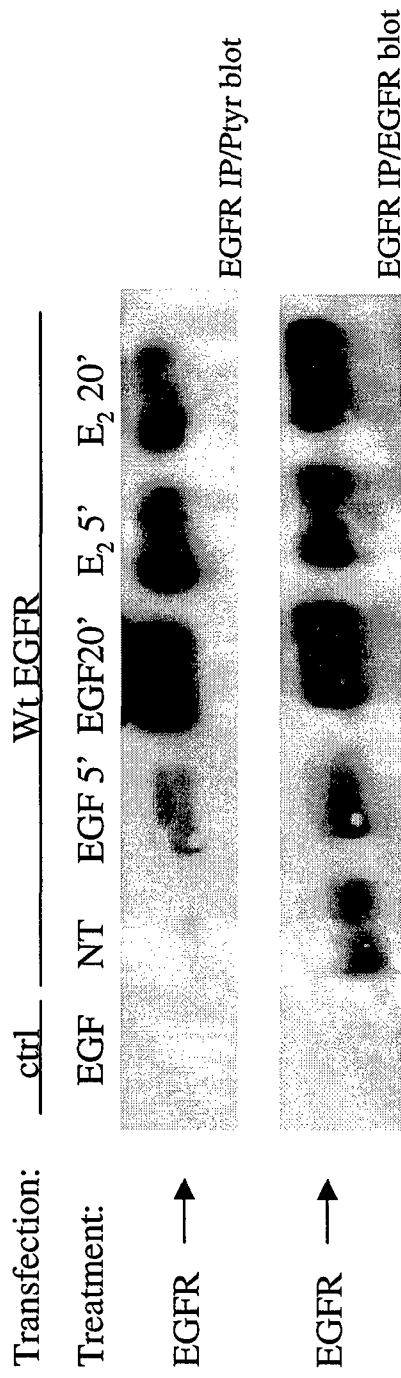


Figure 5. Estradiol induces tyrosyl phosphorylation of the EGFR. MCF7 breast cancer cells were maintained in phenol-red free, charcoal-stripped serum containing media prior to transfection with wild-type EGFR. Cells were allowed to recover for 24 hr then serum-starved overnight. Cells were then stimulated for the indicated times with either EGF or estradiol, lysed in RIPA buffer, and subjected to immunoprecipitation with the F4 EGFR-specific antibody. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, then blotted with either RC20 P_{Tyr} antibody (top panel) or F4 EGFR antibody (bottom panel).

Phosphorylation of EGFR Tyr 845 is Required for EGF and Estrogen Induced Mitogenesis in MCF7 Breast Cancer Cells

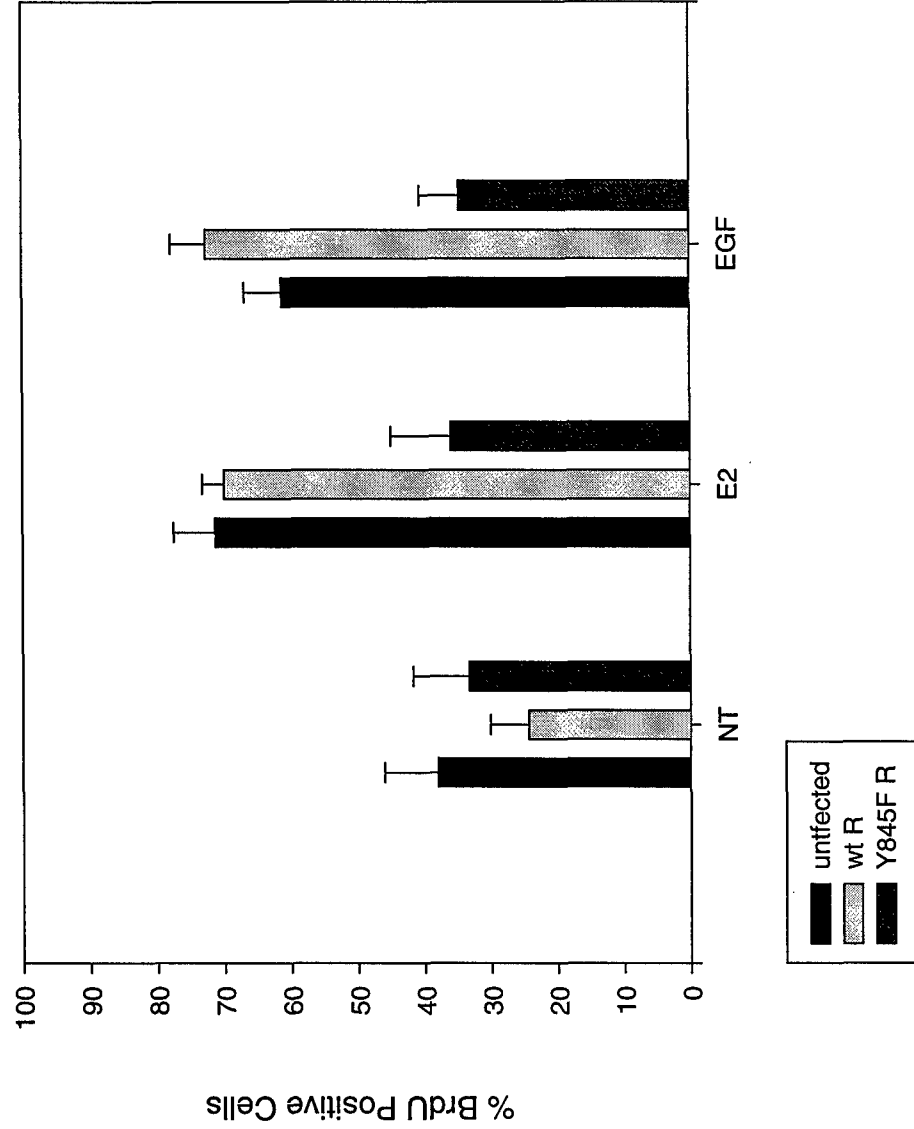


Fig. 6. MCF7 cells maintained in phenol-red free, charcoal stripped serum-containing medium were transfected with plasmid DNA encoding wt or Y845F EGFR, cultured for 24 hr, serum starved for 30 hr, then left untreated or treated with 40 ng/ml EGF or 10 μ M estradiol for 19 hr. Results are expressed as the mean percent \pm SEM of cells expressing the EGFR that were positive for BrdU incorporation. 35-50 cells were analyzed for each variable in 3 independent experiments.

Y845F Mutant EGFR Interferes with EGF-Induced Phosphorylation of STAT5b in K+ Fibroblasts

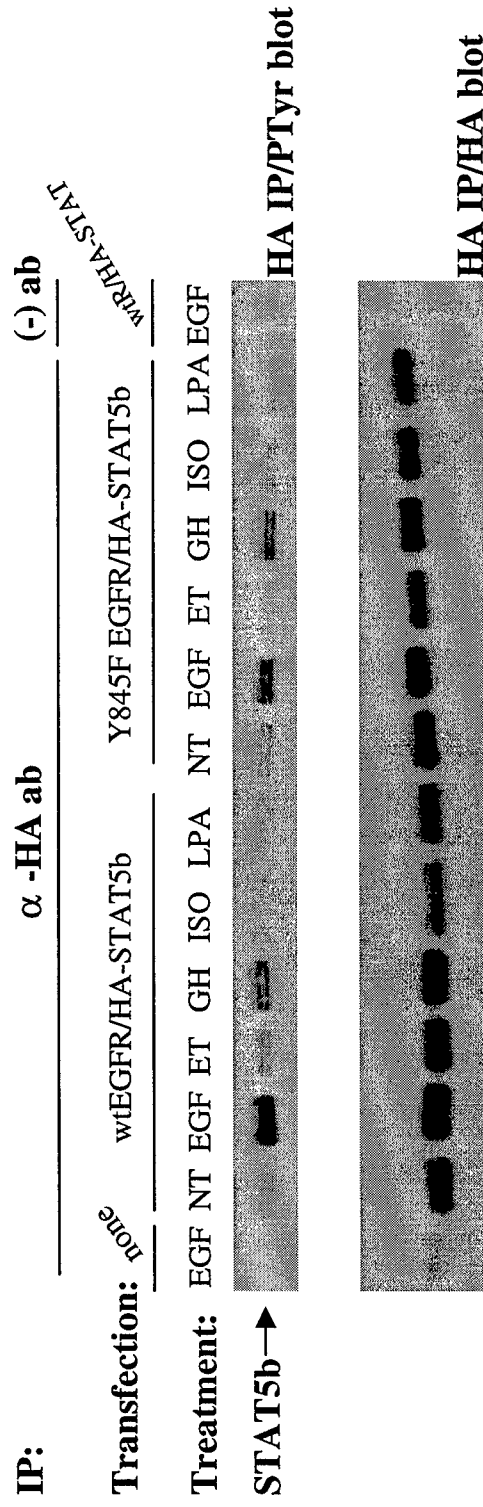


Fig. 7. K+ Src cells were transiently transfected with HA-tagged STAT5b and either wt or Y845F mutant EGFR, stimulated for 10 minutes with the various agonists, lysed in RIPA buffer, and subjected to immunoprecipitation using anti-HA antibodies. Tyrosine phosphorylated STAT5b was visualized using RC20 phosphotyrosine antibody, while overall STAT5b was visualized with the anti-HA antibody. Experiment shown is representative of three independent experiments.

Dominant Negative STAT5b Decreases EGF- and GH- Induced BrdU Incorporation

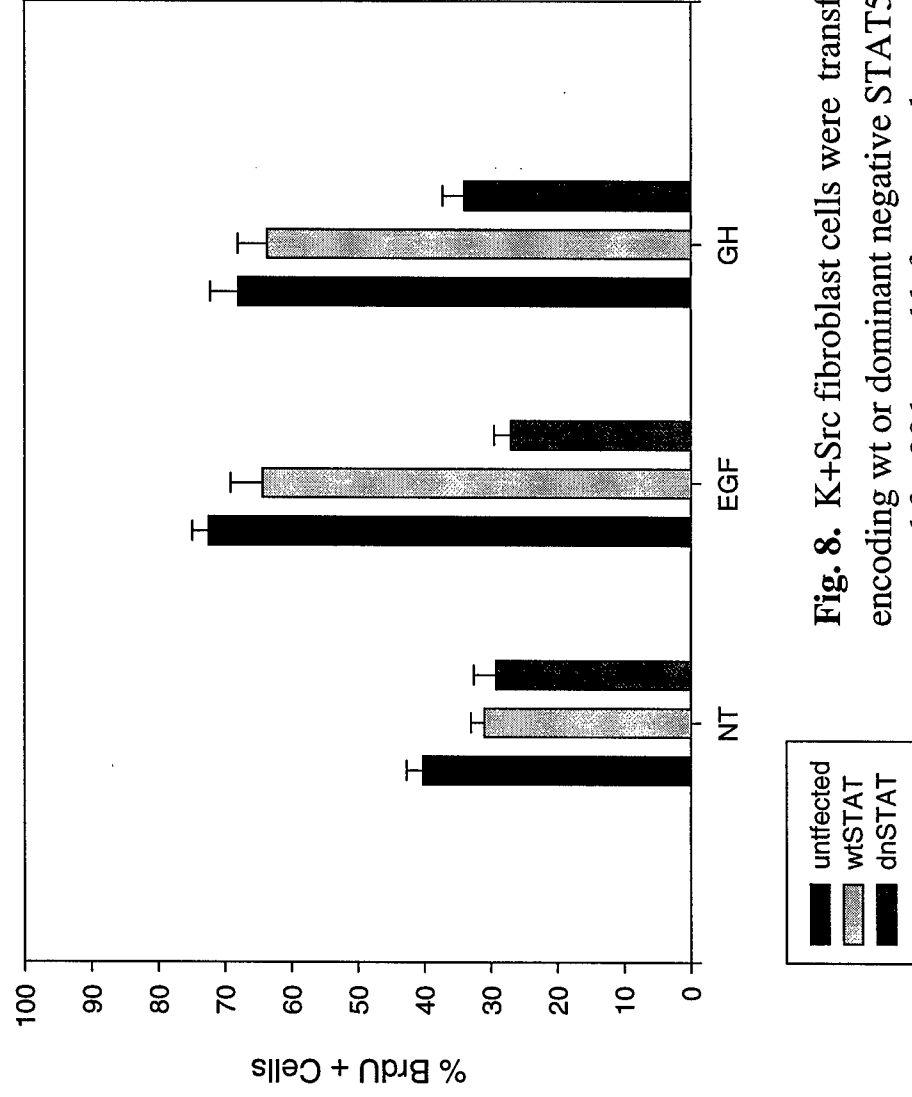


Fig. 8. K+Src fibroblast cells were transfected with plasmid DNA encoding wt or dominant negative STAT5b, cultured for 24 hr, serum starved for 30 hr, and left untreated or treated with agonist for 18 hr. Results are expressed as the mean percent \pm SEM of cells expressing the EGFR that were positive for BrdU incorporation. 35-50 cells were analyzed for each variable in at least 3 independent experiments.

Characterization of Human Epidermal Growth Factor Receptor and c-Src Interactions in Human Breast Tumor Cells

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In C3H/10T1/2 murine fibroblasts, overexpression of both c-Src and the human epidermal growth factor (EGF) receptor 1 (HER1) is required for detection of stable complexes between the two molecules and results in hyperactivation of the receptor and synergistic increases in tumor formation in nude mice, as compared with cells that overexpress only one of the pair. Elevated levels or activities of c-Src and HER1 also occur in a subset of later-stage breast cancers, suggesting that interactions between these two molecules could contribute to a more aggressive clinical course. To determine whether stable complexes between c-Src and HER1 occur in human breast cancers under the same conditions as in murine fibroblasts and whether the appearance of such complexes correlates with enhanced signaling through the EGF receptor and increased tumor growth, human breast tumor cell lines and tumor tissues were analyzed for a number of c-Src/HER1-mediated signaling events and tumorigenicity. In a panel of 14 cell lines, 10 overexpressed c-Src, and of these, five contained elevated levels of HER1 and exhibited an EGF-dependent association between HER1 and c-Src. This association was also present in a HER1/c-Src-overexpressing tumor sample from a breast cancer patient. Further analysis of signaling events revealed that phosphorylation of the HER1 substrate, Shc, and its downstream effector, mitogen-activated protein kinase, was increased in EGF-stimulated MDA-MB-468, MDA-MB-231, and BT-549 cells (which overexpress both c-Src and HER1) as compared with MCF7 and ZR-75-1 cells (which only overexpress c-Src). Furthermore, MDA-MB-468 and MDA-MB-231 cells displayed increased tumorigenicity in nude mice. These results support the hypothesis that c-Src/HER1 interactions contribute to tumor progression in certain late-stage breast tumor cells. *Mol. Carcinog.* 21:261-272, 1998. © 1998 Wiley-Liss, Inc.

Key words: HER1, c-Src, breast cancer, tumorigenesis

INTRODUCTION

Although a variety of effective treatments exist for early-stage, noninvasive breast cancer, large, metastatic tumors or recurrent tumors often fail to respond to conventional radiation and chemotherapy. Thus, investigations into molecular interactions that are specific to later stages in breast cancer progression are important for future drug design. One potential target is the human epidermal growth factor receptor (HER1), overexpression of which is associated with a poor prognosis [1,2]. Increased expression of HER1 has been shown to occur in glioblastomas and carcinomas of the prostate, bladder, kidney, stomach, ovary, and lung [3-7] as well as in breast cancer.

HER1 belongs to a family of receptor tyrosine kinases that includes HER2/neu, HER3, and HER4 [8]. Amplification or overexpression of the genes encoding one or more of the HER family members is estimated to occur in approximately 67% of human breast cancers [9]. Overexpression of HER1 or HER2 protein occurs in 20-30% of human breast tumors, and overexpression of HER1 correlates with loss of estrogen responsiveness [2,7,10-13], suggesting that epidermal growth factor (EGF)-mediated pathways

may be involved in the progression to a more aggressive stage. HER2/neu is located mainly in the primary tumor mass and in earlier stage in situ carcinomas [14-19], which suggests that this molecule is involved in controlling the earlier stages of breast cancer. In contrast, HER1 has been shown to occur at higher levels in metastatic sites than in primary tumors [2,13,20], and it induces an invasive phenotype when expressed in noninvasive rat mammary epithelial cell lines [21,22]. Moreover, overexpression of HER1 protein in a murine fibroblast model leads to transformation when cells are grown in the continuous presence of EGF, demonstrating the potential of HER1 as an oncogene [23].

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Abbreviations: HER, human epidermal growth factor receptor; EGF, epidermal growth factor; PLC γ , phospholipase C γ ; MAPK, mitogen-activated protein kinase; Mab, monoclonal antibody; ER, estrogen receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgG, immunoglobulin G.

Taken together, these findings point to a role for HER1 in the later stage of breast cancer.

Expression or activity of the non-receptor tyrosine kinase c-Src is also increased in a multitude of human tumors, including carcinomas of the breast, stomach, ovary, colon, and prostate [24–27]. In a study by Ottenhalff-Kalff et al. [26], 100% of the primary human breast tumors examined displayed increased overall tyrosine kinase activity, and 70% of the cytosolic tyrosine kinase activity was due to c-Src. Other evidence also indicates that c-Src is involved in the genesis or progression of human breast cancer. Our earlier work demonstrated that overexpression of c-Src potentiates EGF-dependent DNA synthesis, and this effect, as well as normal responsiveness to EGF, is dependent on the presence of a functional c-Src kinase domain [28,29]. This finding was later substantiated by Roche et al. [30]. c-Src has also been shown to physically associate with HER1 and HER2 in certain human and murine breast carcinoma cells [31–33]. That HER1 and c-Src are overexpressed in many of the same tumor types, together with the dependence of EGF signaling on c-Src kinase, suggests that c-Src and HER1 may interact in some manner to potentiate tumorigenesis.

To investigate this possibility, we created a panel of C3H/10T1/2 mouse fibroblasts that overexpress c-Src or HER1 or both. Only in cells overexpressing both HER1 and c-Src was a dramatic and synergistic increase seen in EGF-induced DNA synthesis, colony formation in soft agar, and tumor growth in nude mice [34]. HER1 from the cells overexpressing both HER1 and c-Src formed an EGF-dependent heterocomplex with c-Src and, when complexed with c-Src, became phosphorylated on two previously unidentified tyrosyl residues [34]. The presence of the novel receptor phosphorylations correlated with the hyperphosphorylation on tyrosine of two HER1 substrates, Shc and phospholipase C γ (PLC γ), [35–37], whose tyrosyl phosphorylations were enhanced in both extent and duration in cells overexpressing HER1 and c-Src [34]. These results suggested that phosphorylation of HER1 on one or both of these sites could increase its kinase activity. This increased activity in turn may affect signaling through downstream targets and result in enhanced cell growth. Taken together, these findings indicate that in a fibroblast model system, c-Src and HER1 can act synergistically to promote tumorigenesis.

To assess the possibility that c-Src and HER1 cooperate in a similar fashion during the genesis of human breast cancer, a panel of breast tumor cell lines and tissue samples was examined to determine the levels of c-Src and HER1 protein, the presence of heterocomplexes between the two tyrosine kinases, the levels of tyrosyl phosphorylation on downstream signaling targets, and the presence of tumorigenesis. Rather than conduct an exhaustive survey of all known breast cancer cell lines, we chose to focus on a few representatives (14 cell lines). Some

of these cell lines express receptors for both estrogen and progesterone and are thus thought to represent an earlier stage in the progression of breast cancer, whereas others are negative for these receptors and are thought to represent later disease stages. In this study we demonstrated that of the 14 cell lines examined, five overexpressed both HER1 and c-Src, and only in these five could heterocomplex formation be detected. Cell lines exhibiting an association between c-Src and HER1 also had higher levels of tyrosyl phosphorylation of the HER1 substrate Shc, enhanced activation of mitogen-activated protein kinase (MAPK) in response to EGF, and increased rates and extents of tumorigenicity when compared with cell lines that did not overexpress HER1 or that overexpressed only c-Src. These results are consistent with those from the murine fibroblast model and support the hypothesis that there are functional consequences of overexpressing both c-Src and HER1 that do not accompany overexpression of a single component. These data also demonstrate that double overexpression occurs in a subset of more aggressive breast tumor cells, suggesting that c-Src/HER1 interactions may contribute to increased tumorigenicity of these cells.

MATERIALS AND METHODS

Cell Lines

The derivation and characterization of the clonal C3H/10T1/2 murine fibroblast cell lines used in this study, Neo (control), NeoR1 (an HER overexpressor), and 5HR11 (a c-Src and HER overexpressor) have been described previously [28,29,34]. 5HR11 cells express approximately 25-fold levels of c-Src and NeoR1 and 5HR11 cells express nearly equal levels of cell-surface receptors (approximately 2×10^5 receptors per cell, or about 40-fold over elevated). C3H/10T1/2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, antibiotics, and G418 (400 μ g/mL). The tumor cell lines MDA-MB-468, MDA-MB-231, MCF7, and ZR-75-1 were obtained from N. Rosen (Memorial Sloan Kettering, New York, NY), and the other cell lines were purchased from American Type Culture Collection (Rockville, MD). The tumor cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Where indicated, confluent populations were serum-starved overnight and stimulated for the indicated times with 100 ng/mL purified mouse EGF (Sigma Chemical Co., St. Louis, MO).

Antibodies

Q9 antibody, which was kindly provided by M. Payne, Auburn University, Auburn, AL, was obtained from rabbits immunized with the C-terminal peptide of c-Src (residues 522–533) and exhibited a higher affinity for c-Src than for other Src family members [38,39]. Other c-Src antibodies used were monoclonal

antibody (Mab) 2-17, directed against amino acids 2-17 (Quality Biotech, Camden, NJ) and Mab GD11, directed against the SH3 domain [40,41]. EGF-receptor-specific mouse Mabs 3A and 4A were provided by D. McCarley and R. Schatzman of Syntex Research, Palo Alto, CA. The derivation of Mabs 3A and 4A has been described previously, and their epitopes have been mapped to residues 889-944 and 1052-1134, respectively [34]. Anti-phosphotyrosine polyclonal antibody (RC20) was purchased from Transduction Laboratories (Lexington, KY) and Shc polyclonal antibodies were purchased from UBI (Lake Placid, NY). An anti-estrogen receptor (ER) Mab directed against residues 495-595 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-phospho-MAPK antibody was obtained from Promega Corp. (Madison, WI) and the anti-pan-MAPK antibody B3B9 was the gift of M. Weber (University of Virginia, Charlottesville, VA) [42]. The negative control antibodies included pooled and purified normal rabbit or mouse immunoglobulin (Jackson ImmunoResearch Labs., West Grove, PA).

Immunoprecipitation, Western Immunoblotting, and In Vitro Kinase Assays

The methods for immunoprecipitation, western immunoblotting, and in vitro kinase assays have been described previously [28,29,34]. Cells were lysed in either CHAPS detergent buffer (10 mM CHAPS; 50 mM Tris HCl, pH 8.0; 150 mM NaCl; 2 mM EDTA; 1 mM sodium orthovanadate; 50 µg/mL leupeptin; and 0.5% aprotinin) or in RIPA detergent buffer (150 mM NaCl; 50 mM Tris HCl, pH 7.2; 0.25% sodium deoxycholate; 1% NP-40; 1 mM sodium orthovanadate; 50 µg/mL leupeptin; and 0.5% aprotinin). The tumor samples were prepared by mincing the tissue with a scalpel blade followed by Dounce homogenization in CHAPS buffer. Protein levels were determined by the BCA assay (Pierce Chemical Co., Rockford, IL). For the kinase assays, immunoprecipitates were prepared in and washed twice in CHAPS buffer, followed by one additional wash with HBS buffer (150 mM NaCl and 20 mM HEPES, pH 7.4). Each kinase reaction was conducted in a volume of 20 µL containing 20 mM PIPES, pH 7.5; 10 mM MnCl₂; and 10 µCi of [γ -³²P]ATP (6000 Ci/mM, New England Nuclear Nuclides & Sources Div., Boston, MA) for 10 min at room temperature. The incubations were terminated by the addition of sample buffer, and labeled products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography. In the western immunoblotting procedure, binding of the primary murine or rabbit antibodies to the nitrocellulose membranes was detected with ¹²⁵I-labeled goat anti-mouse immunoglobulin G (IgG), ¹²⁵I-protein A (used at 1 mCi/mL, specific activity 100 µCi/mL (NEN, DuPont Diagnostic Imaging Div., North Billerica, MA) or by enhanced chemiluminescence (Amersham Intl., Plc., Amersham, Bucks, England).

Enolase Assays

c-Src was immunoprecipitated from C3H/10T1/2 fibroblasts and breast tumor cells by using a pool of the anti-Src Mabs GD11 and 2-17, as described above. Two micrograms of acid-treated enolase was added to the washed immunoprecipitates, and the reactions proceeded for 10 min at room temperature, as described by Piwnicka-Worms et al. [43]. To normalize the amount of c-Src precipitated from each cell line, c-Src immunoprecipitates were immunoblotted with 2-17.

Nude Mice Studies

Two million cells were injected subcutaneously into each hindquarter of 6- to 7-wk-old female nude mice (nu/nu; Charles River Laboratories Inc., Wilmington, MA). For each cell line studied, four or five mice were injected, producing a total eight to ten potential tumor sites per cell line. Tumor sizes were measured weekly with microcalipers. Tumor volume was calculated with the formula $V = L \times W^2 \times 0.5$, where L is length and W is width in millimeters.

RESULTS

In Vivo Association of c-Src and HER1 in Human Breast Tumor Cell Lines

To determine whether the c-Src/HER1 synergism model derived from the C3H/10T1/2 fibroblast system is relevant to human breast cancer, 14 breast tumor cell lines were first examined for their expression levels of HER1 and c-Src by immunoblotting. Figure 1 shows that five of these cell lines, SK-BR-3, MDA-MB-468, BT-549, MDA-MB-231, and BT-20 (lanes 4, 7, 10, 11, and 12, respectively) contained elevated levels of HER1 protein when compared to a "normal" control cell line (Hs 578Bst, an immortalized, non-tumorigenic breast epithelial cell line; lane 14). Nine of the cell lines (MDA-MB-175, SK-BR-3, MDA-MB-361, MDA-MB-468, ZR-75-1, BT-549, MDA-MB-231, BT-20, and MCF7; lanes 1, 4, 5, 7, 8, 10, 11, 12, and 13, respectively) and MDA-MB-415 (Table 1) exhibited increased amounts of c-Src protein. The relative levels of HER1 and c-Src were determined by densitometry, and the results are summarized in Table 1. Interestingly, when the same cell lines were examined for levels of HER2, HER1 and HER2 overexpression appeared to be mutually exclusive (data not shown). The potential significance of this finding is discussed below.

To determine whether c-Src and HER1 formed heterocomplexes in the various cell lines, c-Src was immunoprecipitated with an anti-C terminal c-Src antibody from cells mock-treated or stimulated with EGF. The resulting immunoprecipitates were examined for the presence of coprecipitating HER1 with immune complex kinase reactions, as described previously for C3H/10T1/2 cells [34]. After 1 min of stimulation of the 5HR11 cells (included as a positive control) with EGF, a band of 170 kDa was seen

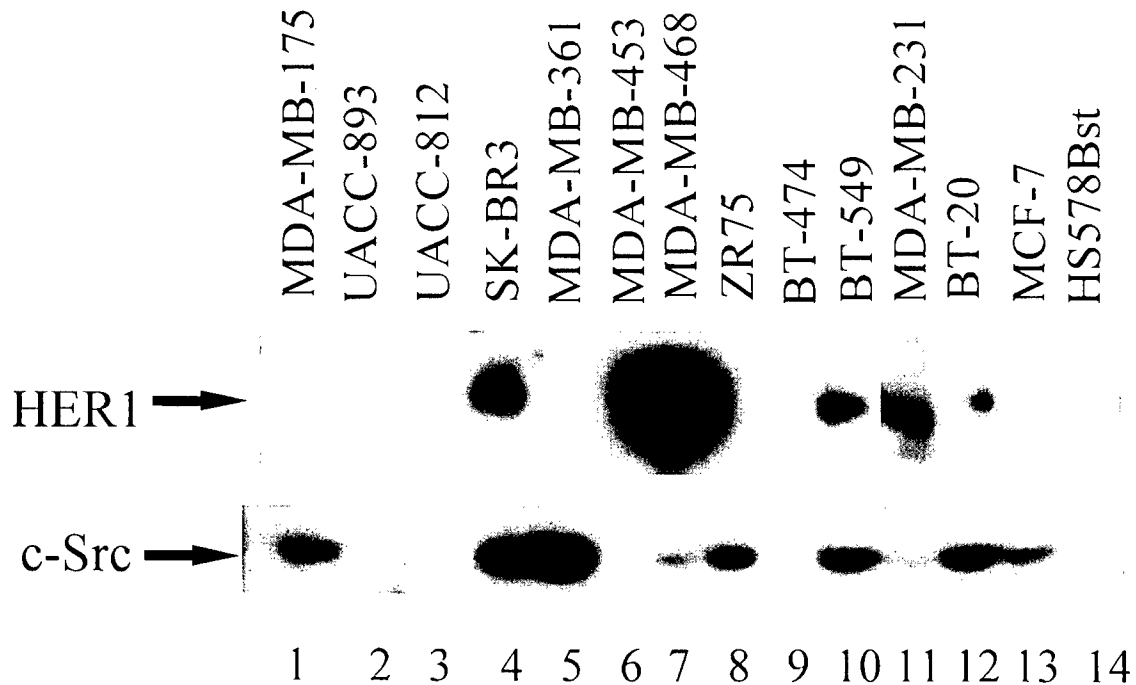


Figure 1. Expression level of HER1 and c-Src in a panel of human breast tumor cell lines. Protein extract (80 μ g) from each cell line was separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with antibodies to HER1 (3A and

4A, top panel) and c-Src (2-17, bottom panel), and detected with 125 I-goat anti-mouse IgG. After longer exposure, c-Src and HER1 bands were visible in the Hs 578Bst lane.

Table 1. Relative Levels of HER1 and c-Src in Human Breast Carcinoma Cell Lines*

Cell line	HER1	c-Src	ER	c-Src/HER1 association
MDA-MB-175	—	9.9	++	—
UACC-893	—	—	—	ND
UACC-812	—	—	—	—
SK-BR-3	12.6	19.4	+	+
MDA-MB-361	—	37.4	+	—
MDA-MB-453	—	—	—	—
MDA-MB-468	39.5	4.9	—	+
ZR-75-1	—	6.2	++	—
BT-474	—	—	+	—
BT-549	6.9	13.4	—	+
MDA-MB-231	7.6	2.9	—	+
BT-20	3.4	13.5	—	+
MCF7	1	6.4	++	—
MDA-MB-415	—	7.6	ND	—
Hs 578Bst [†]	1	1	+	—

—, below limits of detection. Estrogen receptor relative levels were also determined by immunoblotting. —, no expression; +, some expression; ++, strong expression; ND, not determined.

*Relative levels of HER1 and c-Src were determined by densitometry. The lowest detectable signal, in Hs 578Bst cells, was arbitrarily assigned a value of 1, and all other values were expressed relative to this signal. The immunoblot shown in Figure 1 is a representative of several experiments and was chosen to show the varying levels of signals in all the cell lines.

[†]Normal human breast epithelial cells used as negative control.

to co-precipitate with c-Src (Figure 2A, lane 2). The intensity of this band dramatically increased after 10 min stimulation (lane 3) and was greater than the negative antibody control, which was also prepared after 10 min of EGF stimulation (lane 4). We determined that the 170-kDa band from lane 3 corresponded to HER1 by excising it from the gel and comparing its tryptic map with that of non-c-Src-associated HER1, and then the band immunoprecipitated with anti-HER1 antibody from C3H/10T1/2 cells overexpressing HER1 alone (NeoR1 cells; data not shown). In MDA-MB-468, MDA-MB-231, and BT-549 cells, which overexpressed HER1, a 170-kDa phosphoprotein comigrating with HER1 also coprecipitated with c-Src in an EGF-dependent manner (Figure 2B, lanes 1, 3, and 6). This protein was also confirmed to be HER1 by phosphotryptic mapping. An analysis of the additional cell lines revealed that a c-Src/HER1 heterocomplex also existed in SK-BR-3 and BT-20 cells (Table 1) but not in MCF7 or ZR-75-1 cells (Figure 2B, lanes 8–11) or in any of the other remaining cell lines, regardless of whether they overexpressed c-Src. Thus, the association between HER1 and c-Src was observed only in those cell lines that overexpressed both HER1 and c-Src, as in the mouse fibroblast model.

Interestingly, four of the five cell lines that were positive for c-Src/HER1 interaction were also ER nega-

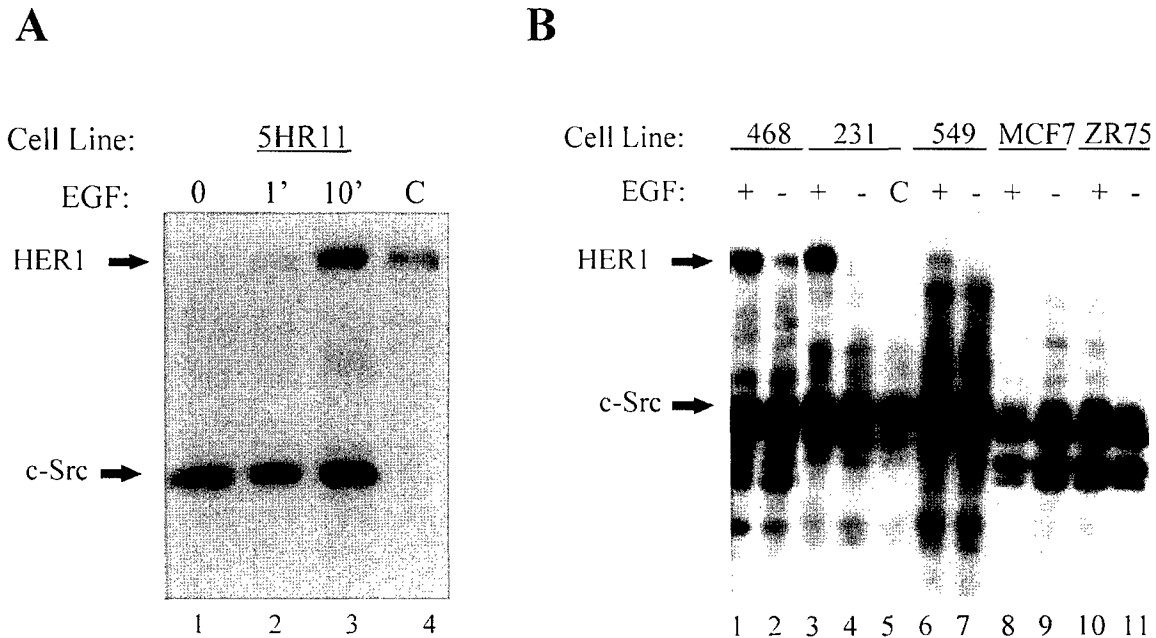


Figure 2. In vivo association between HER1 and c-Src in C3H/10T1/2 cells overexpressing c-Src and HER1 (5HR11 cells) (A) and in human breast cancer cell lines (B). Equal amounts (500 μ g) of cell lysate (in CHAPS detergent) prepared from unstimulated cells or cells stimulated with 100 ng/mL EGF for the indicated times in panel A and for 10 min in panel B were immunoprecipitated with either a c-Src-specific antibody (Q9) or a non-specific control antibody (C). Control immunoprecipitations (panel A, lane 4; panel B, lane 5) were prepared from cells stimulated for 10 min with EGF. Immunocomplexes were then washed and subjected to an in vitro kinase reaction with [γ - 32 P]ATP.

Labeled proteins were resolved by SDS-PAGE and visualized by autoradiography. The background bands in the c-Src region in the control antibody lane of panel B are of unknown origin. c-Src migrated slower than them, as indicated. Within each cell line, stimulated or unstimulated, equal amounts of c-Src protein were immunoprecipitated, as determined by immunoblotting with 2-17, the c-Src specific antibody (data not shown). This experiment was repeated five times with nearly identical results. Other cell lines were also analyzed in the same manner. All results are summarized in Table 1.

tive, as revealed by immunoblotting with an antibody directed to the C terminus of the ER (Table 1). No HER1 was observed to coprecipitate with c-Src from MCF7 and ZR-75-1 cells (Figure 2B, lanes 8 and 10) or from BT-474, MDA-MB-361, and MDA-MB-175 cells (Table 1), which overexpress c-Src but not HER1 and are positive for ER expression. While the ER status of the primary tumors from which these cell lines originated is not known, there does appear to be a correlation between ER negativity and c-Src/HER1 heterocomplex formation in the cell lines.

Immunoprecipitating c-Src with different antibodies, either an Mab directed to the N terminus of c-Src (amino acids 2-17) or one directed to the SH3 domain of c-Src (GD11), also resulted in coprecipitation of HER1 from MDA-MB-468 and MDA-MB-231 cells but not from MCF7 or ZR-75-1 cells (data not shown). In breast tumor cell lines, numerous other bands were seen to coprecipitate with c-Src and to be labeled in the kinase reaction, but the 170-kDa band was the only one that was present in an EGF-dependent, antibody-specific manner.

To see if c-Src/HER1 heterocomplexes could also be detected in tumors, biopsy samples from primary human breast tumors were obtained from patients at the University of Virginia and analyzed as de-

scribed above. Of the three tumor samples depicted, one from a patient with recurrent grade III adenocarcinoma (#52) exhibited elevated expression of HER1 and c-Src as revealed by immunoblotting (Figure 3A). Interestingly, c-Src immunoprecipitates from tumor #52 also showed a band comigrating with HER1 at 170 kDa, indicating that a c-Src/HER1 heterocomplex existed in this particular tumor (Figure 3B). The presence of this complex in tumor #52 was not dependent on addition of exogenous EGF, suggesting that an autocrine mechanism, perhaps involving transforming growth factor α , may be operative in this tumor. A c-Src/HER1 heterocomplex was not observed in the samples of normal breast tissue, in which c-Src expression was low and HER1 expression was undetectable by western immunoblotting, or in the remaining two tumor samples, in which the c-Src level was high but receptor expression was low.

The data from both the cell lines and biopsy samples are consistent with our earlier findings in C3H/10T1/2 cells, whereby we demonstrated that overexpression of both c-Src and HER1 was required to observe complex formation. The only exception in the study presented here is that we were unable to find an example of a human breast cancer cell

ATumor Tissue: 40 52 263 nml

HER1 →



HER1 blot

c-Src →



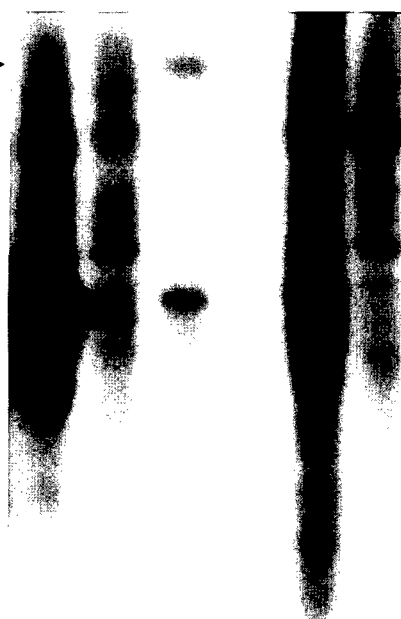
c-Src blot

BTumor Tissue: 40 52 263 normal

IP ab: Src (-) Src (-) Src (-) Src (-)

HER1 →

c-Src →



c-Src IP/kinase

Figure 3. (A) Expression levels of HER1 and c-Src in a panel of human breast tumor tissues and normal breast tissue (nml). Protein extracts (80 μ g) from the various tissues were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with HER1-specific antibodies 3A and 4A (top panel) and c-Src antibody 2-17 (bottom panel), followed by detection with 125 I-anti-mouse IgG. (B) In vivo association

between HER1 and c-Src in human breast tumor tissue. Tissue samples were homogenized in CHAPS detergent, and equal amounts of lysate (500 μ g) were immunoprecipitated with either c-Src antibody Q9 or a nonspecific antibody (-). The immunocomplexes were washed and subjected to an in vitro kinase reaction with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Labeled proteins were resolved by SDS-PAGE and visualized by autoradiography.

line or tumor tissue that overexpressed the receptor but not c-Src. This finding suggests that overexpression of c-Src may precede overexpression of HER1 in the etiology of human breast tumors. For the remaining studies, we chose to focus on five representative cell lines: three that were "positive" for c-Src/HER1 interactions (MDA-MB-468, MDA-MB-231, and BT-549) and two that were "negative" for c-Src/HER1 interactions (MCF7 and ZR-75-1).

Activity of c-Src in Tumor Cell Lines

The ability of c-Src to form a heterocomplex with HER1 may be dependent on the increased expression of c-Src or on the elevation of c-Src-specific kinase activity. To determine whether c-Src kinase activity was altered, five breast tumor cell lines were assayed for the relative specific kinase activity in c-Src immunoprecipitates by using acid-denatured enolase as a substrate. Figure 4 shows that all the tumor

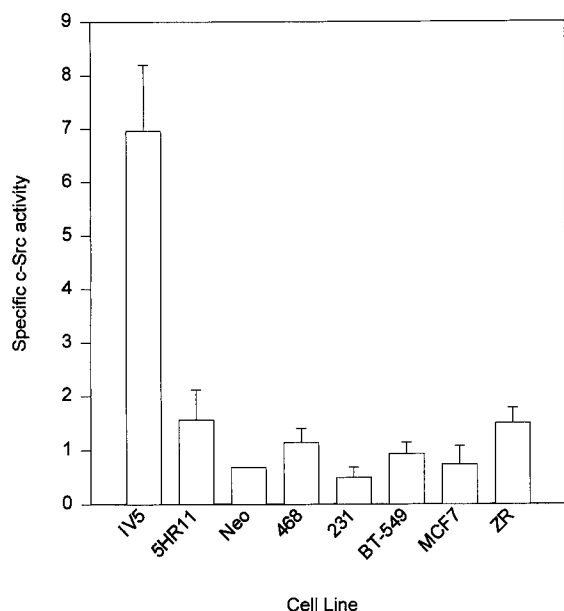


Figure 4. Specific activity of c-Src in C3H/10T1/2 murine fibroblasts and human breast tumor cell lines. c-Src was immunoprecipitated from 500 μ g of RIPA cell lysate by using a pool of Mabs 2-17 and GD11. Two micrograms of acid-treated enolase and 10 μ Ci of [γ - 32 P]ATP were added to the RIPA-washed immunoprecipitates, and kinase reactions were performed in a PIPES/MnCl₂ buffer as described in Materials and Methods. Labeled proteins were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography. c-Src, immunoprecipitated from an equal amount of lysate from each cell line, was immunoblotted with antibody 2-17 and detected with 125 I-labeled rabbit anti-mouse IgG. The amount of radiolabel incorporated into enolase and the amounts of c-Src in the precipitates were quantitated by densitometry, and the specific activities of c-Src were calculated by dividing the densitometric level of enolase phosphorylation by the amount of c-Src immunoprecipitated from each cell line. Except for the Neo cells, the results shown are the averages of three to five independent experiments per cell line.

cell lines examined displayed approximately equal levels of c-Src-specific activity, which was not significantly different from that found in normal fibroblast controls (Neo cells) or in the fibroblasts overexpressing HER1 and c-Src (5HR11). However, Src activity was clearly elevated in the v-Src-transformed cell line IV5, which was included as a positive control. In addition, no consistent changes in c-Src kinase activity were observed when cells were stimulated with 100 ng/mL of EGF for 10 min (data not shown). Therefore, in all the cell lines examined, the ability of c-Src and HER1 to associate with one another correlated with an increase in c-Src protein rather than an increase in specific kinase activity.

Downstream Effectors of HER1 in Breast Tumor Cells

One possible mechanism for the tumorigenicity of these cell lines is enhanced signaling through the increased phosphorylation of HER1 substrate proteins. Previous work from our laboratory demon-

strated that C3H/10T1/2 cells overexpressing both HER1 and c-Src show an increased extent and duration of tyrosyl phosphorylation on the HER1 substrates Shc and PLC γ , as compared with that seen in cells overexpressing either c-Src or HER1 alone [34]. Thus, the synergistic increase in mitogenicity and tumorigenicity observed in these double-overexpressing fibroblasts correlated with the enhanced signaling observed through Shc- or PLC γ -mediated pathways. To determine if elevated c-Src and HER1 expression and heterocomplex formation in breast tumor cells correlated with increased signaling through effectors downstream of the receptor, the tyrosyl phosphorylation states of Shc and PLC γ were examined by immunoprecipitation with specific antibodies followed by immunoblotting with phosphotyrosine-specific antibodies. No tyrosyl phosphorylation of PLC γ was found in response to EGF in any of the five cell lines tested (data not shown). However, Figure 5 shows that in MDA-MB-468, MDA-MB-231, and BT-549 cells, the 46- and 52-kDa isoforms of Shc were highly phosphorylated on tyrosine in response to 10 min of EGF stimulation, whereas MCF7 and ZR-75-1 cells contained significantly lower amounts of tyrosyl-phosphorylated Shc, even when the slight differences in amounts of Shc in the precipitates were taken into consideration. Examining the time course of EGF stimulation confirmed that Shc phosphorylation in MCF7 cells was low at all time points examined between 2 and 30 min (data not shown). Thus, high levels of Shc tyrosyl phosphorylation correlated with overexpression of HER1 and complex formation with c-Src. Whether overexpression of HER1 or complex formation with c-Src is responsible for high levels of Shc phosphorylation cannot be deduced from this analysis, but the findings are consistent with the fibroblast model, which indicated that c-Src and HER1 can cooperate to augment mitogenic signal transduction through certain downstream substrates. These results also suggest that HER1 and c-Src may signal through both PLC γ and Shc in fibroblasts, whereas in epithelial tumor cells, signaling through Shc or other substrates may predominate.

To investigate the EGF-dependent signaling events that are further downstream, the activation state of MAPK was determined by using an antibody specific to the phosphorylated (and therefore activated) form of MAPK. Figure 6 shows that MAPK activity was increased after stimulation with EGF only in those cell lines that displayed c-Src/HER1 interactions (MDA-MB-468, MDA-MB-231, and BT-549 cells). Furthermore, MAPK activation in MDA-MB-231 cells was present even in the absence of EGF stimulation, perhaps because of activation of another component of the HER1-mediated signaling pathway independent of Shc, such as activated ras. In comparison, MCF7 and ZR-75-1 cells contained little or no detectable levels of activated p42 MAPK. As with Shc, MAPK

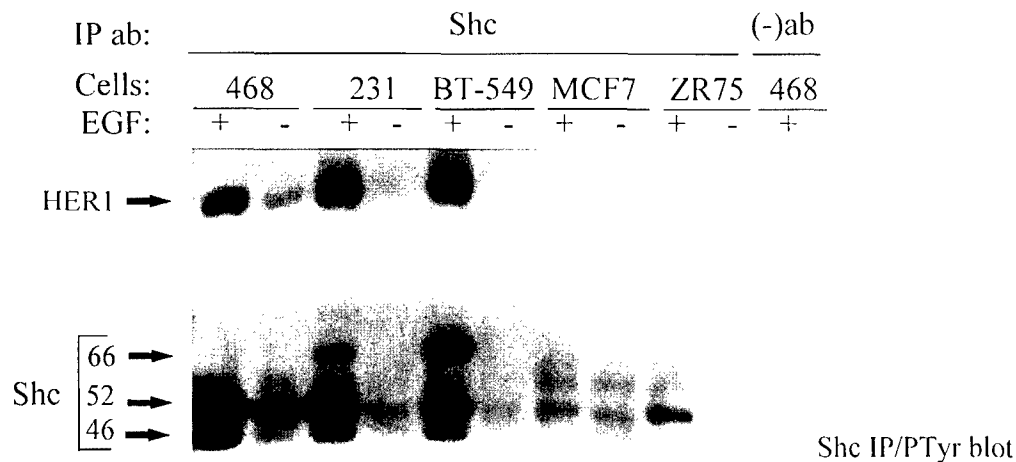
A**B**

Figure 5. Elevated levels of Shc tyrosyl phosphorylation in breast tumor cell lines overexpressing HER1 and c-Src. Lysates (500 µg) were prepared from unstimulated MDA-MB-468, MDA-MB-231, BT-549, MCF7, and ZR-75-1 cells and cells stimulated with 100 ng/mL EGF for 10 min, and extracts were immunopre-

cipitated with either Shc antibody or a nonspecific control antibody ((-)ab) and immunoblotted with pTyr antibody (A) and Shc antibody (B), with enhanced chemiluminescence as the detection method. This experiment represents one of five, all of which yielded similar results.

activation correlates with the detection of c-Src/HER1 heterocomplexes in breast tumor cell lines overexpressing HER1, but one cannot determine whether complex formation or HER1 overexpression is responsible for the high levels of MAPK activity in these cell lines.

Comparative Tumorigenicity of Cell Lines

To determine whether a correlation between c-Src/HER1 association and enhanced tumorigenesis existed, the tumorigenicity of breast cancer cell lines in nude mice was examined. Control C3H/10T1/2 cells overexpressing HER1 alone (NeoR1 cells) and both HER1 and c-Src (5HR11 cells) and the tumor cell lines (MDA-MB-468, MDA-MB-231, BT-549, MCF7, and ZR-75-1) were each injected subcutaneously into the flanks of nu/nu female mice. Tumor growth was measured every week for 7–8 wk. No exogenous estrogen was provided in order to assess the contribution of estrogen-dependent pathways to tumor growth, including the EGF receptor pathway. Table 2 shows that, in a manner similar to that seen in our earlier studies, all the mice injected with the 5HR11 cells developed large, rapidly growing tumors that reached a size of 290 mm³ within 28 d of injection. In contrast to this,

by day 50, only 33% of the mice injected with NeoR1 cells developed tumors, and each tumor was very small, measuring less than 1 mm in diameter. Previous studies have also shown that cells overexpressing c-Src alone fail to develop tumors greater than 1 mm in diameter [34]. Thus, in C3H/10T1/2 cells, overexpression of c-Src in a background of high HER1 expression resulted in a faster growth rate and a more aggressive phenotype than did overexpression of c-Src or HER1 alone.

Nude mice injected with MDA-MB-468, MDA-MB-231, and ZR-75-1 cells also developed tumors that ranged from 10 to 16 mm³ after 50 d. A relatively small number of cells (2×10^6 cells/injection site) was used, so that the rates of tumor growth could be more accurately measured. Only 22% of the mice injected with MCF7 cells formed tumors, which were 1 mm³. The tumorigenic response of the BT-549 cells was also weak, which was unexpected because their EGF-dependent signal-transduction pathways behaved in a manner similar to those of MDA-MB-468 and MDA-MB-231 cells. With respect to ZR-75-1 cells, only 67% of the sites injected developed tumors (compared to 100% and 90% for MDA-MB-231 and MDA-MB-468 cells, respectively), and the initial rate of growth of the ZR-75-1-induced tumors was slower

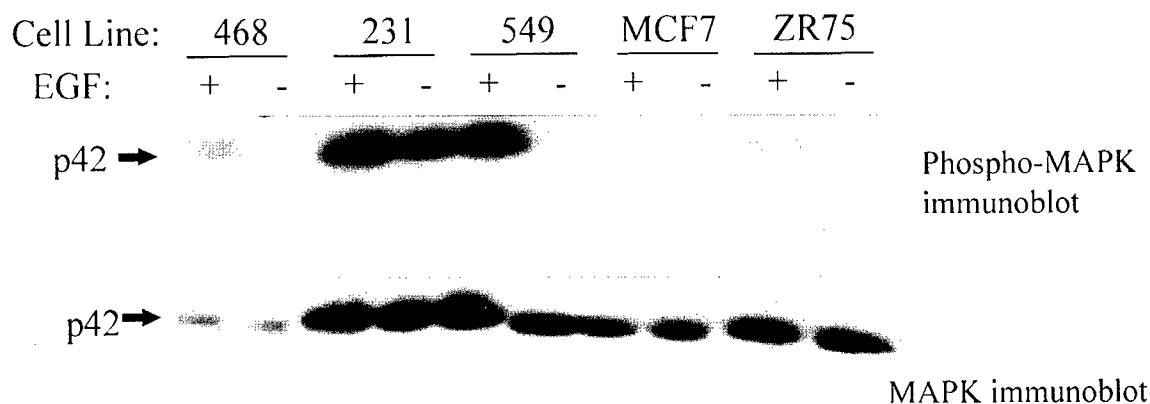


Figure 6. Activation of MAPK in breast tumor cell lines overexpressing HER1 and c-Src. The proteins in 75 μ g of lysate from the indicated cell lines, each of which had been treated with or without 100 ng/mL EGF for 2 min, were separated by

SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either phospho-MAPK antibody or pan-MAPK antibody (B3B9). This experiment was repeated three times with nearly identical results.

than that of the MDA-MB-468 and MDA-MB-231-induced tumors. Thus, these data suggest that c-Src/HER1-mediated signaling pathways could contribute to the tumorigenicity of MDA-MB-468 and MDA-MB-231 cells, whereas different mechanisms may be dominant in the MCF7, ZR-75-1, and BT-549 cells.

DISCUSSION

Here we provide evidence supporting the hypothesis that interactions between c-Src and HER1, shown to result in synergistic increases in mitogenicity and tumorigenicity in fibroblasts, may be operative in human breast cancer as well. In the panel of 14 breast cancer cell lines examined, the ability to detect a heterocomplex between HER1 and c-Src correlated with overexpression of both HER1 and c-Src kinase. The presence of this heterocomplex was EGF dependent, inversely correlated with ER expression, and directly correlated with higher levels of Shc and MAPK activation or phosphorylation. While EGF-induced activation of c-Src was not evident in these cell lines, the increases in Shc and MAPK phospho-

rylation suggest that hyperactivation of the receptor may be a functional consequence of the interaction between c-Src and HER1, as was demonstrated previously in the fibroblast model system [34]. Two of three of the cell lines exhibiting the c-Src/HER1 heterocomplex in this sample produced slightly larger, faster growing, and more numerous tumors in nude mice than did the cell lines that did not overexpress HER1 or show c-Src/HER1 association. Thus, enhanced tumor growth could be mediated in part by a signaling pathway involving HER1, c-Src, Shc, and MAPK.

Other studies have also provided support for the hypothesis that c-Src and HER family members can form heterocomplexes in various tumor cells. Luttrell et al. [31] demonstrated that in MDA-MB-468 cells, c-Src coprecipitates with a 170-kDa phosphotyrosine-containing protein, whereas Sato et al. [44] showed that c-src and HER1 associate in an EGF-dependent manner in HER1-overexpressing A431 cells. c-Src and its related family member HER2 coprecipitate in mammary carcinoma cells derived from HER2/*neu* transgenic mice [32], and the isolated c-Src SH2 domain can bind HER2 immunoprecipitated from SK-BR-3 breast tumor cells [31]. Whereas the experiments in this study did not address the question of whether c-Src binds HER1 directly, other work from our laboratory indicates that these two molecules can interact directly in vitro in a far western assay (Biscardi JS, Maa M-C, Leu T-H, Cox ME, Parsons SJ, submitted for publication) and Muthuswamy and Muller [32] demonstrated direct binding between c-Src and HER2. Other data from our laboratory indicate that c-Src and HER2 also formed a heterocomplex in MDA-MB-361 cells, which express high levels of HER2 (Belsches A and Parsons S, unpublished data). No HER1/c-Src interactions have been detected in this cell line. Interestingly, in the 14 breast tumor cell lines examined in the study reported here, overexpression of HER1 and HER2 appeared to be mutually exclusive, suggesting that within

Table 2. Tumor Formation in Nude Mice*

Cell line	Day 28		Day 50	
	% with tumors	Mean size (mm ³)	% with tumors	Mean size (mm ³)
NeoR1	11	<1	33	<1
5HR11	100	290	—	—
MDA-MB-468	80	14	90	16
MDA-MB-231	57	12	100	10
BT-549	10	1	10	1
ZR-75-1 [†]	67	7	67	13
MCF7	11	<1	22	1

*For each cell line, four or five mice, two subcutaneous injection sites per mouse, and 2×10^6 cells/site were used. —, mice were killed after 35 d because of the large size of the tumors.

[†]In mice injected with ZR-75-1 cells, a single site developed an aberrantly large tumor not included in the table.

any one cell type c-Src binds the most abundantly expressed HER family member.

In contrast to our findings, Stover et al. [33] found that c-Src and HER1 can associate in MCF7 cells, despite the extremely low amounts of HER1 present in these cells. We have been unable to detect HER1 in MCF7 cells, either by immunoblotting or immunoprecipitation with HER1-specific antibodies (unpublished results). While immunoprecipitations with a c-Src antibody directed against the unique domain (amino acids 2–17) did occasionally coprecipitate a 170-kDa band in these cells, this result was not reproducible. Thus, such a heterocomplex may occur in MCF7 cells but be difficult to detect because of the low amount of receptor expressed. Alternatively, the different results could be due to heterogeneity in the MCF7 cell line itself.

The high levels of tyrosyl phosphorylation of the HER1 substrate Shc observed in those breast cancer cells in which HER1 was overexpressed and associated with c-Src could be the result of several different mechanisms: (i) elevated HER1 expression alone may be responsible for the phosphorylation; (ii) c-Src may phosphorylate Shc itself; (iii) c-Src may in some way activate HER1 tyrosine kinase activity; or (iv) another unidentified tyrosine kinase may be activated by HER1 and c-Src and subsequently phosphorylates Shc. While the elevated Shc phosphorylation observed could be due solely to the high levels of HER1 expressed in these cells, two pieces of evidence suggest that alternative mechanisms may exist. First, in the C3H/10T1/2 system, levels of Shc tyrosine phosphorylation are much higher in the 5HR11 fibroblasts overexpressing HER1 and c-Src than in NeoR1 cells, which overexpress HER1 alone [34]. Second, we have found that overexpression of c-Src in MDA-MB-468 cells results in a further EGF-dependent increase in Shc tyrosyl phosphorylation, over and above that seen in the parental MDA-MB-468 cells (Biscardi, et al., submitted for publication). Thus, the increased Shc phosphorylation observed in these breast cancer cells coincides with overexpression of HER1 and c-Src and the presence of heterocomplexes between these two molecules.

Another possible mechanism that explains the increased signaling through Shc and the increased tumorigenesis in MDA-MB-468 and MDA-MB-231 cells is the activation of c-Src after EGF stimulation and the subsequent phosphorylation of Shc by c-Src. Other investigators have reported that in cells overexpressing HER1, EGF treatment induces an increase in c-Src kinase activity [45,46]. Further, Osherov and Levitzki [45] demonstrated that in cells that overexpress HER1 at extremely high levels, such as A431 carcinoma cells, c-Src is constitutively activated independently of EGF stimulation. However, we have been unable to observe a reproducibly EGF-dependent or -independent activation of c-Src in either the C3H/10T1/2 system or in breast tumor cell

lines. This finding is similar to the results of Campbell et al. [47], who also failed to detect an increase in c-Src-specific activity in ZR-75-1 or MCF7 cells. Furthermore, in the C3H/10T1/2 system, cells overexpressing c-Src alone exhibited lower levels of Shc phosphorylation after EGF stimulation than did cells overexpressing the receptor alone or both the receptor and c-Src. These findings suggest that c-Src alone is not responsible for enhanced Shc phosphorylation, but instead may act in concert with HER1.

Finally, the hypothesis that c-Src activates HER1 is supported by findings from the C3H/10T1/2 system, in which phosphorylation of Tyr 845 in c-Src-associated HER1 correlates with enhanced phosphorylation of receptor substrates [34] (Biscardi et al., submitted for publication). Sequences surrounding this tyrosyl residue are 50% homologous to those surrounding Tyr 416 of c-Src, phosphorylation of which is known to increase Src kinase activity [48]. Another novel phosphorylation (Tyr 1101) of the receptor has been detected in kinase reactions of c-Src-associated HER1 derived from 5HR11 murine fibroblasts in vitro and in vivo and from MDA-MB-468 or MDA-MB-231 cells in vitro (Biscardi et al., submitted for publication). Therefore, the elevated levels of Shc tyrosyl phosphorylation could result from activation of HER1 by phosphorylation at either Tyr 845 or Tyr 1101 or be the result of a more complicated signaling pathway involving heterodimers with other HER family members or with other tyrosine kinases.

The finding that c-Src and HER1 form a heterocomplex in certain breast tumor cell lines suggests that interactions between these two kinases could contribute to a more aggressive tumor phenotype. Indeed, in the panel of cell lines that we tested, there was a general correlation between the presence of increased tumorigenicity in nude mice and HER1/c-Src heterocomplexes. An exception was BT-549 cells, which produced very small tumors despite being positive for c-Src/HER1 interactions. Such a result is not unexpected, given the genetic heterogeneity of tumor cells in general and the multitude of signaling pathways that can contribute to tumorigenicity. Therefore, taken with results from the C3H/10T1/2 system, in which overexpression of c-Src and HER1 results in a synergistic increase in tumor size, these data are consistent with the notion that c-Src and HER1 are critical regulators of the growth of some but not all human breast tumors.

HER1 or HER2 overexpression occurs in 20–30% of human breast tumors and is correlated with poorer prognosis and loss of estrogen responsiveness [2,7,10–13], whereas c-Src expression and activity are elevated in most breast tumors regardless of stage [26,27]. In this regard, four of five cell lines that exhibited c-Src/HER1 interactions were ER negative (Table 1), whereas six of nine cell lines that expressed very little HER1 and did not associate with c-Src were ER positive.

While it is possible that some of the ER-positive cell lines may have nonfunctional receptors, our results still suggest that the presence of c-Src/HER1 complexes may be one indicator of a more aggressive phenotype.

That HER1 and c-Src interact physically and functionally has potentially important therapeutic implications. Neither c-Src nor HER1 is overexpressed in normal breast tissues, suggesting that complex formation and the resulting synergistic signaling occur to a much greater extent in tumor cells than in normal cells. It is possible that interdiction of the signaling events enhanced by c-Src/HER1 interactions could result in decreased cell growth. Reagents that could disrupt the c-Src/HER1 heterocomplex or otherwise interfere with the synergistic signaling between these two molecules could be used therapeutically in a tumor-specific manner. By interdicting an event more specific to tumor cells than to normal cells, the potential side effects and toxicity associated with more general tyrosine kinase inhibitors could be avoided. Thus, studies such as these may provide a basis for the design of novel therapies for breast cancer.

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c-Src-mediated Phosphorylation of the Epidermal Growth Factor Receptor on Tyr⁸⁴⁵ and Tyr¹¹⁰¹ Is Associated with Modulation of Receptor Function*

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Accumulating evidence indicates that interactions between the epidermal growth factor receptor (EGFR) and the nonreceptor tyrosine kinase c-Src may contribute to an aggressive phenotype in multiple human tumors. Previous work from our laboratory demonstrated that murine fibroblasts which overexpress both these tyrosine kinases display synergistic increases in DNA synthesis, soft agar growth, and tumor formation in nude mice, and increased phosphorylation of the receptor substrates Shc and phospholipase γ as compared with single overexpressors. These parameters correlated with the ability of c-Src and EGFR to form an EGF-dependent heterocomplex *in vivo*. Here we provide evidence that association between c-Src and EGFR can occur directly, as shown by receptor overlay experiments, and that it results in the appearance of two novel tyrosine phosphorylations on the receptor that are seen both *in vitro* and *in vivo* following EGF stimulation. Edman degradation analyses and co-migration of synthetic peptides with EGFR-derived tryptic phosphopeptides identify these sites as Tyr⁸⁴⁵ and Tyr¹¹⁰¹. Tyr¹¹⁰¹ lies within the carboxyl-terminal region of the EGFR among sites of receptor autophosphorylation, while Tyr⁸⁴⁵ resides in the catalytic domain, in a position analogous to Tyr⁴¹⁶ of c-Src. Phosphorylation of Tyr⁴¹⁶ and homologous residues in other tyrosine kinase receptors has been shown to be required for or to increase catalytic activity, suggesting that c-Src can influence EGFR activity by mediating phosphorylation of Tyr⁸⁴⁵. Indeed, EGF-induced phosphorylation of Tyr⁸⁴⁵ was increased in MDA468 human breast cancer cells engineered to overexpress c-Src as compared with parental MDA 468 cells. Furthermore, transient expression of a Y845F variant EGFR in murine fibroblasts resulted in an ablation of EGF-induced DNA synthesis to nonstimulated levels. Together, these data support the hypothesis that c-Src-mediated phosphorylation of EGFR Tyr⁸⁴⁵ is involved in regulation of receptor function, as well as in tumor progression.

The epidermal growth factor receptor (EGFR)¹ is a 170-kDa

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¹ The abbreviations used are: EGFR, epidermal growth factor recep-

single-pass transmembrane tyrosine kinase that undergoes homo- or heterodimerization and enzymatic activation following ligand binding (1, 2). These events result in the trans-(auto)-phosphorylation of multiple Tyr residues in the COOH-terminal tail of the molecule that serve as binding sites for cytosolic signaling proteins containing Src homology 2 (SH2) domains (3). Five sites of *in vivo* autophosphorylation have been identified in the EGFR: three major (Tyr¹⁰⁶⁸, Tyr¹¹⁴⁸, and Tyr¹¹⁷³) and two minor (Tyr⁹⁹² and Tyr¹⁰⁸⁶) (4–7). These sites bind a variety of downstream signaling proteins which contain SH2 domains, including Shc (8) and PLC γ (9). Binding of these or other signaling proteins to the receptor and/or their phosphorylation results in transmission of subsequent signaling events that culminate in DNA synthesis and cell division.

c-Src is a nonreceptor tyrosine kinase that functions as a co-transducer of transmembrane signals emanating from a variety of polypeptide growth factor receptors, including the EGFR (see Refs. 10 and 11, and reviewed in Ref. 12). Overexpression of wild type (wt) and dominant negative forms of c-Src in murine C3H10T1/2 fibroblasts that express normal levels of receptor, as well as experiments involving the microinjection of antibodies specific for Src family members, have revealed that c-Src is a critical component of EGF-induced mitogenesis (10, 11, 13). Cells which express high levels of EGFR become transformed upon continual exposure to EGF (14), and co-overexpression of c-Src in these cells dramatically potentiates their growth and malignant properties (15). Together, these findings indicate that c-Src co-operates with the EGFR in the processes of both mitogenesis and transformation.

Subsequent studies in 10T1/2 cells revealed that potentiation of EGF-induced growth and tumorigenesis by c-Src, which is observed only in cells overexpressing both c-Src and the receptor, correlates with the EGF-dependent formation of a heterocomplex containing c-Src and activated EGFR, the appearance of two unique *in vitro* non-autophosphorylation sites on receptors in complex with c-Src, and enhanced *in vivo* tyrosyl phosphorylation of the receptor substrates, PLC γ and Shc (15). These findings suggested that c-Src-dependent phosphorylations on the EGFR can result in hyperactivation of receptor kinase activity, as measured by the enhanced ability of the receptor to phosphorylate its cognate substrates. This report identifies Tyr⁸⁴⁵ and Tyr¹¹⁰¹ as c-Src-dependent sites of phosphorylation, which are present both *in vitro* and *in vivo* in receptor from 10T1/2 double overexpressing fibroblasts and

tor; DMEM, Dulbecco's modified Eagle's medium; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PLC γ , phospholipase C γ ; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; BrdUrd, bromodeoxyuridine; GST, glutathione S-transferase; mAb, monoclonal antibody.

from MDA468 human breast cancer cells. In the MDA468 cells, overexpression of c-Src results in a further increase in the phosphorylation of Tyr⁸⁴⁵, indicating that c-Src either phosphorylates this site directly or activates a secondary kinase which is responsible. Moreover, cells which transiently express EGFR bearing a Tyr to Phe mutation at Tyr⁸⁴⁵ are impaired in their ability to synthesize DNA in response to EGF, suggesting that this c-Src mediated phosphorylation site is important for receptor function.

MATERIALS AND METHODS

Cell Lines—The derivation and characterization of the clonal C3H10T1/2 murine fibroblast cell lines used in this study, Neo (control), 5H (c-Src overexpressor), NeoR1 (human EGFR overexpressor), and 5HR11 (c-Src/EGFR double overexpressor) have been described previously (10, 11, 13). 5H and 5HR11 express equal levels of c-Src (~25-fold over endogenous), and NeoR and 5HR11 express nearly equal levels of cell surface receptors (~2 × 10⁶ receptors/cell or ~40-fold over endogenous). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., Gaithersburg, MD), containing 10% fetal calf serum, antibiotics, and G418 (400 µg/ml). When indicated, confluent cultures were starved of serum overnight, prior to stimulation with 100 ng/ml purified mouse EGF (Sigma).

To create cells transiently overexpressing HER1 which contained a Tyr to Phe mutation at position 845, a *Dra*III-*Bst*EII fragment from pCO11 (gift of Laura Beguinot), including the mutation at position 845, was subcloned into a pcDNA vector containing wild type human HER1 (gift of Dr. Stuart Decker, Parke Davis, Ann Arbor, MI). Neo control 10T1/2 fibroblasts were transiently transfected with 30 µg of Superfect (Qiagen, Chatsworth CA) and 4 µg of vector, wt HER1, or Y845F HER1 plasmid DNA according to the manufacturers' directions and incubated for 48 h.

For overexpression of c-Src in breast cancer cells, pcDNAc-Src was constructed by inserting the c-Src *Xho*I fragment from an existing pVZneo vector into the multicloning site of pcDNA3 (Invitrogen, San Diego, CA). MDA468 cells, obtained from N. Rosen (Sloan Kettering Cancer Center, New York), were maintained in DMEM plus 5% serum. MDA468 cells stably overexpressing chicken c-Src (clone MDA468c-Src) were generated by LipofectinTM (Life Technologies, Inc.)-mediated gene transfer of pcDNAc-Src into parental MDA468 cells and selection with 400 µg/ml G418. Parental MDA468 cells overexpress c-Src approximately 5-fold, as compared with Hs578Bst normal breast epithelial cells, and contain approximately 10⁶ receptors/cell (Ref. 16),² while MDA468c-Src cells overexpress c-Src approximately 25-fold over levels found in normal breast epithelial cells.

Antibodies—EGFR-specific mouse monoclonal antibodies (mAbs) 3A and 4A were provided by D. McCarley and R. Schatzman of Syntex Research, Palo Alto, CA. Their derivation has been described previously and their epitopes have been mapped to residues 889–944 and 1052–1134, respectively. EGFR-specific mAb F4, directed against amino acids 985–996, was obtained from Sigma. GD11 antibody is directed against the SH3 domain of c-Src and was characterized previously in our laboratory (17, 18). Q9 antibody was raised in rabbits against the COOH-terminal peptide of c-Src (residues 522–533) and exhibits a higher affinity for c-Src than for other Src family members (19, 20). Antiphosphotyrosine (Tyr(P)) antibody (4G10) was purchased from UBI (Lake Placid, NY). Negative control antibodies included pooled and purified normal rabbit or mouse immunoglobulin.

Immunoprecipitation, Western Immunoblotting, and in Vitro Kinase Assays—Methods for immunoprecipitation, Western immunoblotting, and *in vitro* kinase assays have been described previously (10, 11, 15). Cells were lysed either in CHAPS detergent buffer (10 mM CHAPS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 µg/ml leupeptin, and 0.5% aprotinin), or in RIPA detergent buffer (0.25% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 50 µg/ml leupeptin, and 0.5% aprotinin). Protein concentrations of detergent lysates were determined by the BCA protein assay (Pierce, Rockford, IL). 500 µg of cell lysate was used for immunoprecipitations, and 50 µg was used for Western blotting. For Western immunoblotting, binding of primary murine or rabbit antibodies to Immobilon membranes was detected with either ¹²⁵I-labeled goat anti-mouse IgG (NEN Life Science Products Inc.) or ¹²⁵I-protein A (NEN) used at 1 µCi/ml, specific activity 100 µCi/ml. For

kinase assays, immunoprecipitates were prepared in and washed twice in CHAPS buffer, then washed twice with HBS buffer (150 mM NaCl, 20 mM HEPES, pH 7.4). Each kinase reaction was conducted in 20-µl volumes containing 20 mM PIPES, pH 7.5, 10 mM MnCl₂, and 10 µCi of [³²P]ATP (6000 Ci/mmol, NEN) for 10 min at room temperature. Incubations were terminated by addition of sample buffer, and labeled products were resolved by SDS-PAGE and visualized by autoradiography.

In Vitro Binding and Far Western Analysis Using GSTc-SrcSH2—The construction and preparation of GST fusion proteins containing the SH2 domain of c-Src was described previously (21). To reconstitute binding between tyrosyl-phosphorylated EGFR and the SH2 domain of c-Src, 2 µg of immobilized GST-c-SrcSH2 fusion protein was incubated with 100 µg of 10T1/2 cell lysate protein prepared in RIPA buffer. After 3 h gentle mixing at 4 °C, beads were washed three times with RIPA buffer, resuspended in SDS sample buffer, and boiled. Eluted proteins were separated by SDS-PAGE, transferred to Immobilon, and immunoblotted with either the Tyr(P) or the 3A/4A or F4 (Sigma) monoclonal EGFR antibodies.

To assess direct binding of GST-c-SrcSH2 to the EGFR, receptor from 500 µg of cell lysate protein in RIPA buffer was immunoprecipitated with 3A/4A mAbs. The resulting EGFR immunoprecipitates were resolved by SDS-PAGE, transferred to Immobilon membranes, and incubated with 1 mg/ml purified GST-c-SrcSH2 fusion protein in blocking buffer at 4 °C overnight. The membrane was then probed with 1 µg/ml affinity purified, polyclonal rabbit anti-GST antiserum in blocking buffer,³ and immunoglobulin binding was detected by ¹²⁵I-protein A.

Metabolic Labeling—NeoR1 or 5HR11 cells were grown to 50–75% confluency in 150-mm dishes, washed with phosphate-free DMEM, and incubated for 18 h in phosphate-free DMEM containing 0.1% dialyzed fetal bovine serum and 1 mCi/ml [³²P]orthophosphate (NEN Life Science Products Inc.) in a final volume of 10 ml. For pervanadate treatment, labeling medium was adjusted to a concentration of 3 mM H₂O₂ and 5 µM Na₂VO₄ just prior to EGF stimulation. Cells were stimulated in the presence of pervanadate by addition of 100 ng/ml EGF to the labeling medium for 5 min, washed twice with phosphate-free DMEM, and lysed in CHAPS detergent buffer. Extract from an entire plate (approximately 1–2 mg of protein) was immunoprecipitated with c-Src or EGFR-specific antibodies as described above.

Two-dimensional Tryptic Phosphopeptide Analysis—Immunoprecipitates of *in vitro* or *in vivo* ³²P-labeled EGFR were resolved by SDS-PAGE. The EGFR was localized by autoradiography, excised from the gel, and digested with trypsin as described by Boyle *et al.* (22). Phosphotryptic peptides were separated by electrophoresis at pH 1.9 in the first dimension and ascending chromatography in the second dimension on cellulose thin layer chromatography (TLC) plates. Chromatography buffer contained isobutyric acid, 1-butanol, pyridine, acetic acid, H₂O (125:3.8:9.6:5.8:55.8). Migration of synthetic phosphopeptides was detected by spraying the dried TLC plate with a hypochlorite solution consisting of sequential sprays with 10% commercial Clorox, 95% ethanol, 1% potassium iodide, and saturated o-tolidine in 1.5 M acetic acid, as described in Stewart and Young (23).

High Performance Liquid Chromatography (HPLC)—For HPLC analysis of peptides derived from the EGFR associated with c-Src, ³²P-labeled phosphotryptic peptides were prepared as above and suspended in 0.05% trifluoroacetic acid. Peptides were injected into a Perkin-Elmer Series 4 Liquid Chromatograph equipped with a Vydac C18 column (4.6 × 250 mm) and eluted with increasing concentrations of acetonitrile (0 to 100%) at a flow rate of 1 ml/min, as described by Wasilenko *et al.* (24). 500-µl fractions were collected, and Cerenkov counts of each fraction were determined. Fractions containing peptides "0" and "3" were identified by two-dimensional TLC analysis for their ability to co-migrate with the appropriate peptide in a mixture of total *in vitro* phosphorylated receptor peptides. Appropriate fractions were then lyophilized and subjected to Edman degradation.

Edman Degradation—HPLC fractions of ³²P-labeled EGFR phosphotryptic peptides or spots eluted from TLC plates were subjected to automated Edman degradation, as performed by the University of Virginia Biomolecular Research Facility. Briefly, phosphorylated peptides were coupled to a Sequelon aryl amine membrane (25), washed with 4 × 1 ml of 27% acetonitrile, 9% trifluoroacetic acid, and 2 × 1 ml of 50% methanol, and transferred to an applied Biosystems 470A sequenator using the cartridge inverted as suggested by Stokoe *et al.* (26). The cycle used for sequencing was based on that of Meyer *et al.* (27), but modified

² N. Rosen, personal communication.

³ J. H. Chang and S. Parsons, unpublished data.

by direct collection of anilinothiazolinone amino acids in neat trifluoroacetic acid as described by Russo *et al.* (28). Radioactivity was measured by Cerenkov counting.

Identification of Peptides 0 and 3—Phosphorylated peptides (corresponding to residues GMN(Y-P)LEDR, candidate for peptide 3; or E(Y-P)HAEGGK, candidate for peptide 0) were synthesized by the University of Virginia Biomolecular Research Facility. Synthetic peptides were mixed with oxidized *in vitro* labeled phosphotryptic peptides from c-Src-associated EGFR, separated on cellulose TLC plates, and visualized by spraying with the hypochlorite solution as described above. One candidate for peptide 3 (GMNYLEDR) was synthesized as a phosphopeptide and tested for comigration as above. Another candidate for peptide 3 (DPHY¹¹⁰¹QDPHSTAVGNPEYLNTVQPTCVNSTF DSPAH-WAQQ), which was too large to chemically synthesize, was tested by further digestion of *in vitro* labeled peptide 3 with a proline-directed protease (Seikagaku, Rockville, MD), according to the method of Boyle *et al.* (22). In brief, the spot corresponding to peptide 3 was scraped off the TLC plate, eluted with pH 1.9 buffer, and digested with 5 units of proline-directed protease in 50 mM ammonium bicarbonate at pH 7.6 at 37 °C for 1 h. Peptides were separated by two-dimensional electrophoresis as described above.

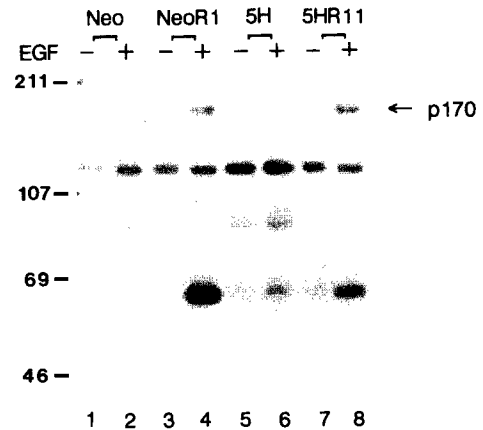
BrdUrd Incorporation—Neo control cells, which had been transfected with cDNAs encoding wild type EGFR, Y845F EGFR, or vector alone were cultured for 48 h, then serum starved for an additional 30 h prior to the administration of 100 μ M BrdUrd and either 100 ng/ml EGF or 10% fetal calf serum in fresh growth medium. Treated cells were incubated for 18 h and co-stained for HER1 expression and BrdUrd incorporation as described by the manufacturer of the BrdUrd-specific antibody (Boehringer Mannheim). Briefly, fixed cells were treated with 2 N HCl for 1 h at 37 °C and incubated with a mixture of primary antibodies (1:100 dilution of the HER1-specific Ab-4, and a 1:15 dilution of anti-BrdUrd mouse antibody in serum-free medium for 1 h at 37 °C), followed by incubation with a mixture of secondary antibodies (75 μ g/ml fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and 4 μ g/ml Texas Red-conjugated goat anti-mouse IgG) for 1 h at 37 °C. Both secondary reagents were obtained from Jackson Immunoresearch Laboratories, West Grove, PA.

RESULTS

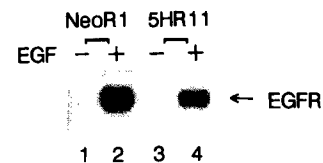
Direct Binding of c-Src SH2 Domain to the EGFR—Previous work from our laboratory demonstrated a synergistic interaction between c-Src and the EGFR which led to increased cell growth and tumor development (10, 11, 15). This functional synergism was most striking when cells overexpressed both c-Src and the EGFR (5HR cells) and correlated with the ability of c-Src and the EGFR to form specific, EGF-dependent hetero-complexes *in vivo*. The formation of this c-Src-EGFR complex raises the question of whether binding between c-Src and the EGFR occurs directly, or is mediated by another protein present in the complex. To test whether association could be mediated by a Tyr(P)-SH2 interaction, lysates from unstimulated and stimulated Neo, 5H, NeoR, or 5HR cells were incubated with a GST-c-SrcSH2 bacterial fusion protein linked to agarose beads, and precipitated proteins were probed with Tyr(P) antibody. Fig. 1, panel A, lanes 4 and 8, show that a tyrosyl-phosphorylated protein of 170 kDa was precipitated by GST-c-SrcSH2 from extracts of cells overexpressing the EGFR after activation of the receptor with EGF. This 170-kDa protein co-migrated with the EGFR precipitated with receptor-specific mAbs 3A/4A (data not shown). Other proteins that bound c-SrcSH2 included p125^{FAK} (21), which was detected in all the cell lysates, a 75–80-kDa protein, cortactin, which was most prominent in 5H cells (30), and a 62-kDa protein, presumed to be related to the 62-kDa “DOK” protein associated with p120^{Ras-GAP} (31–34). These results suggest that *in vivo*, multiple Tyr(P)-containing proteins in addition to the EGFR are capable of interacting with c-Src via its SH2 domain and contribute to the highly tumorigenic phenotype of the double overexpressing cells. Incubation of cell extracts with GST-beads alone resulted in no detectable binding of Tyr(P)-containing proteins (data not shown).

To confirm that the 170-kDa protein was indeed the EGFR,

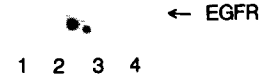
A src-SH2 IP/p-Tyr blot



B src-SH2 IP/EGFR blot



C EGFR IP/src-SH2 blot



D EGFR IP/EGFR blot

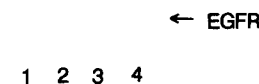


FIG. 1. *In vitro* association between activated EGFR and c-Src SH2 domain. 500 μ g of lysate protein from the indicated nonstimulated cells or cells stimulated with 100 ng/ml EGF for 2 min was incubated with GST-c-SrcSH2 fusion protein immobilized on glutathione-agarose beads (Panels A and B) or EGFR mAbs 3A/4A bound to Protein A (Panels C and D), as described under “Materials and Methods.” Affinity-precipitated proteins were washed and subjected to SDS-PAGE, transferred to Immobilon membranes, and probed with: A, Tyr(P) mAb 4G10; B, EGFR mAbs 3A/4A; C, GST-c-Src SH2 fusion protein; and D, EGFR mAb 3A/4A. Binding of primary antibody was visualized by incubating membranes with ¹²⁵I-labeled goat anti-mouse IgG (Panels A, B, and D), and binding of GST-c-SrcSH2 fusion protein was detected by rabbit anti-GST and ¹²⁵I-protein A (Panel C). GST-c-SrcSH2 fusion protein is shown to bind directly to activated EGFR.

lysates prepared from unstimulated and stimulated NeoR and 5HR cells were precipitated with immobilized GST-c-SrcSH2, and bound proteins were immunoblotted with EGFR-specific mAbs 3A/4A. Fig. 1, Panel B, demonstrates that receptor antibody detected the 170-kDa protein only in stimulated cells, as in Panel A, confirming its identity as the EGFR. To test if the interaction between the activated EGFR and c-SrcSH2 could be direct, receptor immunoprecipitates were subjected to a “Far Western” overlay experiment, using GST-c-SrcSH2, GST-specific antibody, and ¹²⁵I-protein A. Fig. 1, Panel C, lanes 2 and 4, shows that GST-c-SrcSH2 bound the EGFR and, as predicted, the interaction required activation by EGF. GST alone exhibited no binding (data not shown). Panel D verified that nearly equal amounts of receptor were present in all immunoprecipitates. These results provide evidence for the involvement of SH2-Tyr(P) interactions in the formation of the EGFR-c-Src complex.

In Vivo and in Vitro Phosphorylation of Novel, Non-auto-

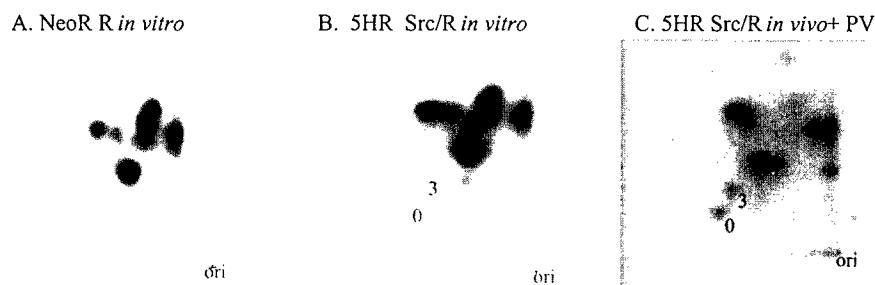


FIG. 2. EGFR phosphotryptic peptides radiolabeled *in vitro* or *in vivo*. For *in vitro* labeling (Panels A and B), 5HR and NeoR cells were stimulated with 100 ng/ml EGF for 30 min, followed by lysis in CHAPS buffer and immunoprecipitation of extract proteins with either c-Src-specific (GD11) or EGFR-specific (3A/4A) antibody. Precipitated proteins were then subjected to an *in vitro* kinase reaction, and products were analyzed by SDS-PAGE and autoradiography. For *in vivo* experiments (Panel C), cells were labeled for 18 h in phosphate-free media containing [³²P]orthophosphate, stimulated with 100 ng/ml EGF for 5 min in the presence of pervanadate, and lysed in CHAPS buffer. Extracts were immunoprecipitated with GD11 antibody, and precipitated proteins were analyzed by SDS-PAGE and autoradiography. c-Src-associated, ³²P-labeled EGFR was eluted from gel slices, and samples were trypsinized and analyzed by two-dimensional TLC as described previously (17). Labeled peptides were visualized by autoradiography. Panel A, *in vitro* labeled EGFR immunocomplexes from NeoR cells (2000 cpm); Panel B, *in vitro* labeled c-Src-associated EGFR from 5HR cells (2000 cpm); Panel C, c-Src-associated EGFR from 5HR cells labeled *in vivo* (3000 cpm). Tryptic maps were exposed to Pegasus Blue film (Pegasus, Burtonsville, MD) for 18 h.

phosphorylation Sites on the EGFR in Complex with c-Src—Overexpression of both EGFR and c-Src in 10T1/2 cells results in increased tyrosyl phosphorylation of receptor substrates, PLC γ and Shc, following EGF treatment (15). These findings suggest that the c-Src-associated receptor is modified in some manner as to increase its kinase activity. To examine the receptor for novel phosphorylations, the *in vitro* phosphorylated, c-Src-associated 170-kDa protein was excised from the gel and subjected to two-dimensional phosphotryptic peptide analysis. The phosphopeptide map of c-Src-associated receptor was then compared with the map of the free receptor, immunoprecipitated with receptor antibody. Fig. 2, Panels A and B, demonstrate that the maps are nearly identical; however, two additional phosphorylations (designated peptides 0 and 3) were seen in the map of the EGFR complexed with c-Src, suggesting that c-Src was responsible for their phosphorylation. Consistent with this notion, two-dimensional phosphoamino acid analysis of the *in vitro* labeled EGFR demonstrated that peptides 0 and 3 contained only phosphotyrosine (data not shown). Panel C shows that the two novel phosphopeptides were also detected in the receptor found in complex with c-Src from ³²P metabolically labeled 5HR cells that had been treated with pervanadate and EGF for 5 min. These data indicate that two phosphorylations occur on the EGFR both *in vitro* and *in vivo* when c-Src becomes physically associated with the receptor following EGF stimulation.

Initial attempts to detect peptides 0 and 3 in receptor immunoprecipitations from ³²P-labeled NeoR or 5HR cells yielded phosphopeptide maps that contained peptide 3 but no or barely detectable levels of peptide 0 (Fig. 3, Panels A and C). Neither could peptide 3 nor peptide 0 be detected reproducibly in receptor that was associated with c-Src from 5HR cells (data not shown). Furthermore, in receptor immunoprecipitations, the levels of peptide 3 derived from NeoR *versus* 5HR cells appeared nearly equal (compare Panels A and C), suggesting that peptide 3 may not be an *in vivo*, c-Src-dependent site of phosphorylation. In these experiments, lysates were prepared in CHAPS buffer containing a mixture of conventional protease and phosphatase inhibitors, including orthovanadate (see "Materials and Methods"). However, modification of the EGF treatment regimen to include pervanadate during stimulation allowed us to detect peptide 0 in receptor immunoprecipitates from NeoR (Panel B) and 5HR (Panel D) cells. These conditions revealed more peptide 0 in receptor from 5HR than from NeoR cells, confirming the ability of c-Src to modulate the phosphorylation of this peptide. Of special note was the finding that peptide 0 was the only peptide seen to increase in phosphoryl-

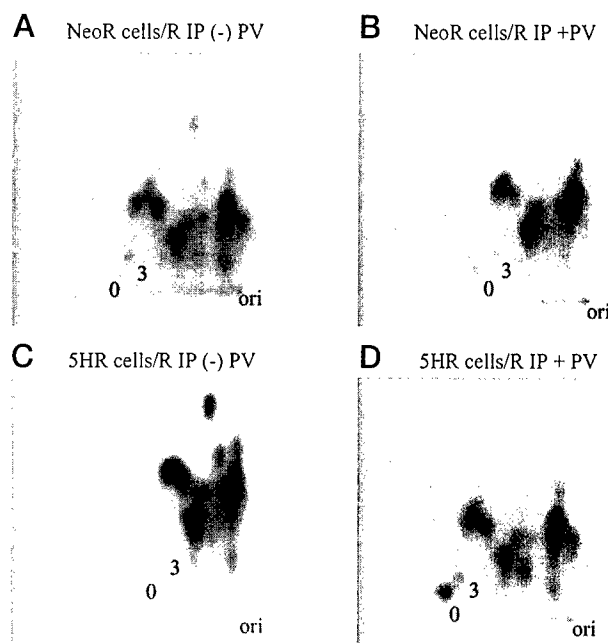


FIG. 3. Phosphorylation of peptides 0 and 3 in metabolically labeled pervanadate-treated cells. NeoR and 5HR cells were incubated for 18 h with [³²P]orthophosphate as above. Pervanadate (3 mM H₂O₂ and 5 μ M Na₂VO₄) was added (Panels B and D) or not (Panels A and C) along with 100 ng/ml EGF for 5 min prior to lysis in RIPA detergent buffer. EGFR was immunoprecipitated with mAbs 3A/4A, and the receptor was processed for phosphotryptic analysis as described in the legend to Fig. 3. Panel A, EGFR from NeoR cells; Panel B, EGFR from pervanadate-treated NeoR cells; Panel C, EGFR from 5HR cells; Panel D, EGFR from pervanadate-treated 5HR cells. ~3000 cpm were loaded per TLC plate. TLC plates were exposed to Pegasus blue film for 18 h.

ation in response to pervanadate treatment, suggesting that its phosphorylation is more labile than that of peptide 3 or the other phosphorylations on the receptor, which presumably correspond to autophosphorylation sites. Together with the *in vitro* studies depicted in Fig. 2, the results from the *in vivo* experiments indicate that peptide 0 is an *in vitro* and *in vivo* site of receptor phosphorylation that is regulatable by c-Src. Following this line of reasoning, the low level of peptide 0 phosphorylation seen in receptor immunoprecipitates from NeoR cells (Fig. 3, Panel B) could be due to endogenous c-Src. However, the involvement of other tyrosine kinases in the *in vivo* phosphorylation of peptide 0 cannot be ruled out.

Whether c-Src alone plays a role in regulating the phospho-

A peptide "0"

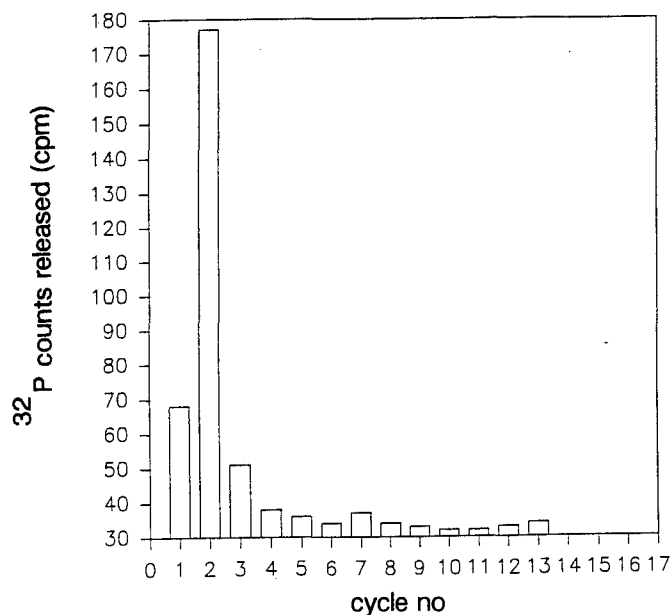
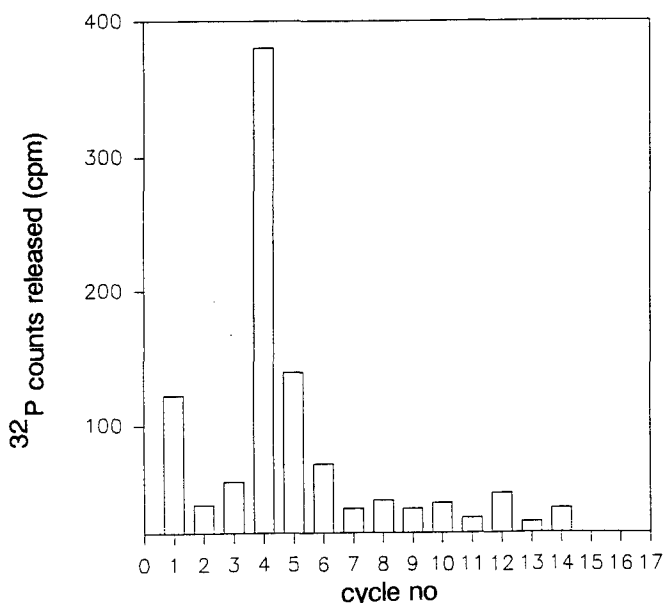


FIG. 4. Edman degradation of peptides 0 and 3. Peptides 0 and 3 were isolated by HPLC and subjected to automated Edman analysis. A, ³²P from peptide 0 was released at the second cycle, indicating a phosphorylated tyrosine at position 2; B, ³²P from peptide 3 was released at the fourth cycle, indicating a phosphorylated tyrosine at position 4.

B peptide "3"



rylation of peptide 3 *in vivo* is less clear. *In vitro*, peptide 3 phosphorylation appears to be unique to the receptor associated with c-Src (compare Panels A and B of Fig. 2), and HPLC analysis corroborates this, where phosphorylation of the peak corresponding to peptide 3 was found to be ~3.5-fold greater when the receptor was associated with c-Src *versus* free receptor (data not shown). Furthermore, the level of *in vivo* phosphorylation of peptide 3 in the c-Src-associated receptor is greater than that found in the "free" receptor (compare Fig. 2, Panel C, with Fig. 3, Panel D). However, peptide 3 is readily detected in free receptor labeled *in vivo*, and its level of phosphorylation does not appear to increase to any great extent in 5HR *versus* NeoR cells (Fig. 3, Panels B and D). These data can be interpreted to mean either that peptide 3 contains a non-labile site of phosphorylation, regulatable by c-Src (in contrast to peptide 0), or that phosphorylation of peptide 3 may be

regulated by an additional tyrosine kinase *in vivo*.

To identify the amino acids phosphorylated *in vitro* in a c-Src-dependent manner, fractions containing peptides 0 and 3 were isolated by HPLC. Peptide 0 eluted at 8.5% acetonitrile, while peptide 3 eluted at 10.5% acetonitrile (not shown). These HPLC fractions, which were of greater than 95% purity, were subjected to sequential Edman degradation to determine the cycle number at which radioactivity was released. Results from these analyses indicated that a phosphoamino acid residue was located at the second position of peptide 0 (Fig. 4, Panel A) and at the fourth position of peptide 3 (Fig. 4, Panel B). Of the tryptic peptides generated from the intracellular domain of the EGFR which contain Tyr residues, those peptides containing Tyr⁸⁴⁵, Tyr⁸⁶⁷, or Tyr⁸⁹¹ were potential candidates for peptide 0, while those peptides containing Tyr⁸⁰³ or Tyr¹¹⁰¹ were potential candidates for peptide 3 (see Table I).

TABLE I
Candidates for peptides 0 and 3

Peptide 0	845 Glu- Tyr -His-Ala-Glu-Gly-Gly-Lys
	867 Ile- Tyr -Thr-His-Gln-Ser-Asp-Val-Trp-Ser-Tyr-Gly-Val-Thr-Val Trp-Glu-Leu-Met-Thr-Phe-Gly-Ser-Lys
	891 Pro- Tyr -Asp-Gly-Ile-Pro-Ala-Ser-Glu-Ile-Ser-Ser-Ile-Leu-Glu Lys
Peptide 3	803 Gly-Met-Asn- Tyr -Leu-Glu-Asp-Arg
	1101 Asp-Pro-His- Tyr -Gln-Asp-Pro-His-Ser-Thr-Ala-Val-Gly-Asn Pro-Glu-Tyr-Leu-Asn-Thr-Val-Gln-Pro-Thr-Cys-Val-Asn-Ser Thr-Phe-Asp-Ser-Pro-Ala-His-Trp-Ala-Gln-Lys

The Tyr⁸⁴⁵-containing peptide was selected for further study as a candidate for peptide 0, since it showed 50% homology to sequences contained within the autophosphorylation site of Src (Tyr⁴¹⁶), indicating that it could be a potential c-Src target. The octamer composed of E(P-Y⁸⁴⁵)HAEGGK (peptide 0) was chemically synthesized to include a phosphorylated Tyr⁸⁴⁵ and analyzed either alone (Fig. 5, Panel A) or in a mixture with total peptides from *in vitro* labeled, c-Src-associated receptor by two-dimensional TLC (Panel C). The synthetic octamer comigrated with peptide 0 in the mixture, thereby identifying Tyr⁸⁴⁵ as the phosphorylated residue in peptide 0.

Since peptides 0 and 3 migrated similarly in the two-dimensional chromatography, it was expected that they would share similar isoelectric points and hydrophobicities. Both candidates for peptide 3 (GMNY⁸⁰³LEDR or DPHY¹¹⁰¹QDPHSTAVGNPEYLNVTQPTCVNSTFDSPAHWAKQ, see Table I) had theoretical isoelectric points and calculated hydrophobic indices (22) similar to those of the Tyr⁸⁴⁵-containing peptide, indicating that both were potential candidates. The Tyr⁸⁰³-containing peptide was selected first for further study, since it was smaller and more easily synthesized. However, this synthetic phosphopeptide did not co-migrate with peptide 3 nor with any of the other EGFR phosphopeptides (data not shown), indicating that the Tyr¹¹⁰¹-containing peptide was the preferred candidate. To verify the identity of peptide 3, *in vitro* labeled peptide 3 was scraped off the TLC plate, eluted with pH 1.9 buffer, and subjected to further digestion with a proline-directed protease as described under "Materials and Methods." Since, of the two candidate peptides, only the Tyr¹¹⁰¹-containing peptide contains proline residues, any change in mobility resulting from digestion with this protease would confirm its identity as peptide 3. As a control, peptide 0, which does not contain any proline residues, was digested with proline-directed protease and no change in mobility was observed (data not shown). Fig. 6 shows that digestion of spot 3 with the proline-directed protease resulted in a change of migration primarily in the first dimension (compare Panel A with Panel B). To confirm that a mobility shift was indeed occurring, digested and undigested peptide 3 were mixed (Panel C). The results identify peptide 3 as Tyr¹¹⁰¹.

Phosphorylation of Tyr⁸⁴⁵ and Tyr¹¹⁰¹ in HER1 from Breast Tumor Cells—Our laboratory has previously demonstrated the presence of EGF-dependent c-Src-EGFR heterocomplexes in several human breast tumor cell lines including MDA468, which overexpresses both c-Src and HER1 (16). Since the presence of this heterocomplex is correlated with general increases in downstream receptor-mediated signaling and tumorigenicity in these cells, as compared with cell lines which do not overexpress the EGFR, we wished to investigate whether Tyr⁸⁴⁵ and/or Tyr¹¹⁰¹ were phosphorylated in c-Src-associated EGFR derived from breast tumor cells. Fig. 7 demonstrates

that phosphopeptides 0 and 3 are both present in *in vitro* labeled, c-Src-associated EGFR from EGF-stimulated MDA468 cells, although peptide 0 is weakly detected in the absence of pervanadate treatment. To further investigate the role of c-Src in mediating the phosphorylation of these sites, an MDA468 derivative cell line which stably overexpresses c-Src approximately 25-fold over levels in normal breast epithelial cells (MDA468c-Src cells, Panel B) was created. In these cells, the phosphorylation of peptide 0 (Tyr⁸⁴⁵) was greatly enhanced, while the phosphorylation of peptide 3 (Tyr¹¹⁰¹) was unchanged (Panel C).

Role of Tyr⁸⁴⁵ in EGF-dependent Mitogenesis—A tyrosyl residue homologous to Tyr⁸⁴⁵ is conserved in many other receptor tyrosine kinases, and mutation of these conserved tyrosines to phenylalanine results in a reduced ability of the receptors to signal downstream events (35–37). Thus, it is possible that mutation of Tyr⁸⁴⁵ to phenylalanine would likewise decrease EGF-dependent signaling through the EGFR. To directly test the requirement of Tyr⁸⁴⁵ phosphorylation for receptor function, a variant receptor bearing a Y845F mutation was transiently transfected into Neo cells, and the effects on DNA synthesis were assayed by measuring bromodeoxyuridine (BrdUrd) incorporation in response to EGF (Fig. 8). The level of BrdUrd incorporation in cells expressing the Y845F mutant EGFR was reduced to approximately 30% of that induced by the wild type receptor, indicating that the mutant EGFR could interfere with the function of endogenous receptor and was thus acting in a dominant negative manner. Similar results were obtained when Y845F receptor was expressed in cells which overexpress c-Src (38). These findings suggest that phosphorylation of Tyr⁸⁴⁵ is necessary for the mitogenic function of the receptor.

DISCUSSION

Previous studies from our laboratory using the C3H10T1/2 murine fibroblast model demonstrated that simultaneous overexpression of c-Src and EGFR potentiates EGF-dependent mitogenesis, transformation, and tumorigenesis, as well as EGF-dependent association of c-Src with the receptor and increases in tyrosyl phosphorylation of the receptor substrates Shc and PLCγ (15). These events correlated with the appearance of two novel tyrosine phosphorylation sites on the receptor, suggesting that one mechanism by which c-Src could synergize with the EGFR is by physically complexing with it and mediating the phosphorylation of novel non-autophosphorylation tyrosine residues, which in turn may result in hyperactivation of the receptor and enhanced phosphorylation of receptor substrates. This increased signaling would then culminate in augmented cell division and tumor growth. Such a model was recapitulated in breast cancer cell lines of epithelial origin, wherein cell lines that express high levels of c-Src and EGFR exhibit EGF-de-

FIG. 5. Identification of peptide 0. The octapeptide E(Y-P)HAEGGK was synthesized to contain phosphorylated Tyr⁸⁴⁵ and analyzed by two-dimensional electrophoresis/chromatography on TLC plates, either alone (*Panel A*) or in a mixture with total *in vitro* labeled tryptic phosphopeptides derived from the receptor which co-precipitated with c-Src (*Panel C*). The synthetic phosphopeptide, detected by hypochlorite spraying, co-migrated with tryptic peptide 0, verifying Tyr⁸⁴⁵ as the site on the receptor whose phosphorylation is dependent on c-Src. *Panel B*, total phosphopeptides from c-Src-associated receptor alone. *Panel D*, sequence homology between the peptide containing Tyr⁴¹⁶ of c-Src and the peptide containing Tyr⁸⁴⁵ of the EGFR. 3000 cpm of *in vitro* labeled tryptic phosphopeptides were loaded along with 2 μ g of synthetic phosphopeptide.

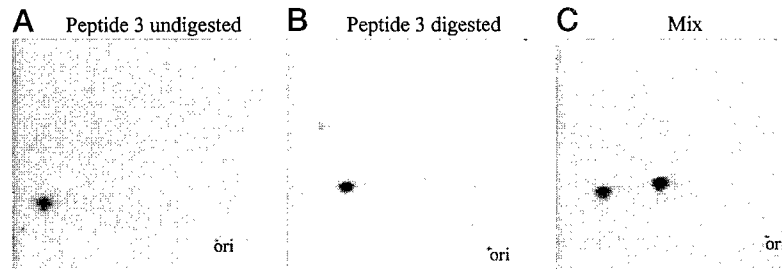
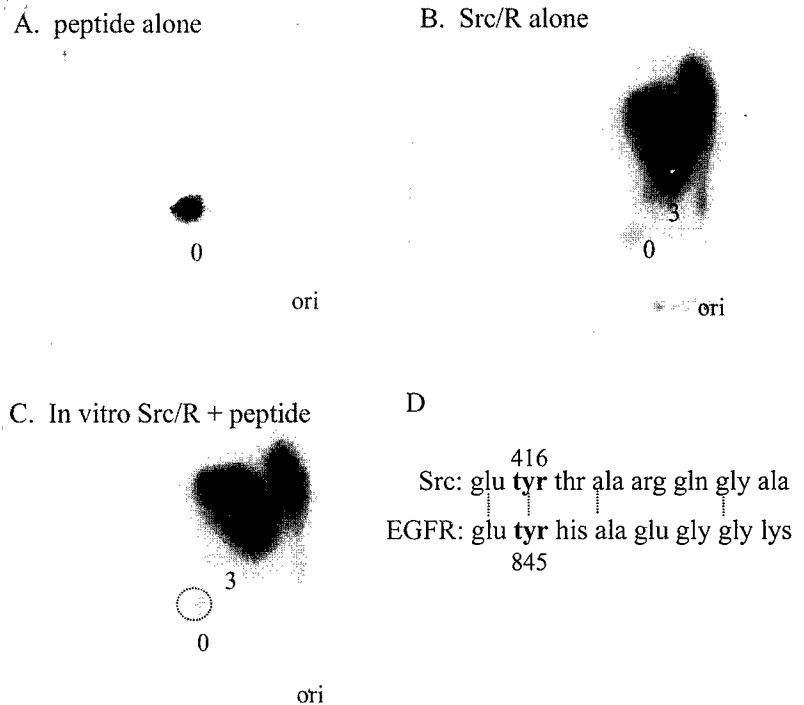


FIG. 6. Identification of peptide 3. *In vitro* phosphorylated peptide 3 (as in Fig. 5B) was scraped and eluted from the TLC plate and subjected to digestion with proline-directed protease. Undigested or digested, eluted peptide 3 was then analyzed by two-dimensional TLC either alone (*Panels A and B*, respectively) or mixed (*Panel C*). The altered mobility of digested peptide 3 indicates the presence of a proline in the sequence and identifies the peptide as containing Tyr¹¹⁰¹. 100 cpm of either digested or undigested peptide 3 were loaded on each TLC plate.

pendent association between c-Src and the receptor, augmented signaling through Shc and MAP kinase, and enhanced tumor formation, as compared with breast tumor cell lines which do not overexpress both c-Src and the EGFR (16). Because these and other studies link c-Src and the EGFR etiologically to tumorigenesis and malignant progression in many human tumors (reviewed in Ref. 12), identification of the two novel c-Src-dependent phosphorylations on the receptor and determination of their functions has taken on added importance, as they represent possible sites for therapeutic intervention.

Here we identify these c-Src dependent sites as Tyr⁸⁴⁵ and Tyr¹¹⁰¹ and demonstrate that they become phosphorylated in murine fibroblasts both *in vitro* and *in vivo* in c-Src/EGFR double overexpressing cells in an EGF-dependent manner. Enhanced phosphorylation of Tyr⁸⁴⁵ was also observed in MDA468 human breast cancer cells when c-Src was overexpressed, indicating that such phosphorylations can occur in cells of both mesodermal and epithelial origin. More importantly, the fact that cells expressing a Y845F variant of the EGFR are impaired in their ability to synthesize DNA in response to EGF treatment provides direct evidence for the importance of this phosphorylation. Together, these findings support the hypothesis that the c-Src-mediated phosphorylation of Tyr⁸⁴⁵ is a critical event for EGFR function, and in certain situ-

ations where overexpression of these molecules exists (such as in certain breast tumors), the increased receptor signaling resulting from this phosphorylation could lead to enhanced tumorigenesis.

Tyr⁸⁴⁵ resides in an intriguing position on the receptor, namely in the activation lip of the kinase domain (39, 40). Amino acid sequences in this lip are highly conserved among tyrosine kinases (41). Crystallographic studies indicate that phosphorylation of Tyr⁸⁴⁵ homologues stabilizes the activation lip, maintains the enzyme in an active state, and provides a binding surface for substrate proteins; while mutation of these sites in their respective receptors results in decreases in cell growth and transformation (37, 40–43). A similar situation appears to exist for the EGFR, as cells expressing the Y845F variant receptor showed decreases in their ability to respond mitogenically to EGF. This impairment of DNA synthesis occurred both in a background of endogenous levels of c-Src, as shown here, as well as in cells where c-Src was overexpressed (38). This finding argues that endogenous levels of c-Src are capable of mediating the phosphorylation of Tyr⁸⁴⁵ and that the Y845F form of the receptor acts in a dominant negative fashion. Which downstream targets of the receptor are affected in various cell types by the Y845F mutation is not known. Other studies from our laboratory demonstrate that EGF-induced increases in Shc and mitogen-activated protein kinase tyrosyl

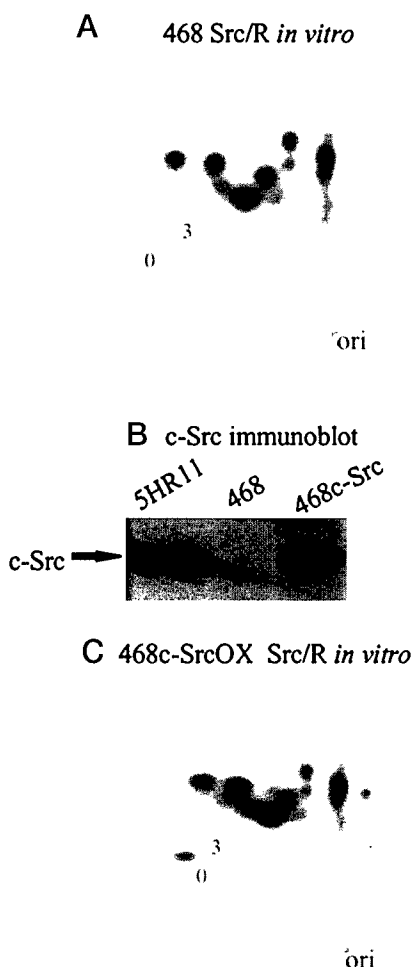


FIG. 7. Phosphorylation of Tyr⁸⁴⁵ and Tyr¹¹⁰¹ in MDA468 breast tumor cells. MDA468 or MDA468c-Src cells were stimulated with 100 ng/ml EGF for 30 min, followed by lysis in CHAPS buffer and immunoprecipitation of extract proteins with either c-Src-specific (GD11) or EGFR-specific (F4) antibody. Precipitated proteins were then subjected to an *in vitro* kinase reaction. The labeled EGFR was eluted from gel slices, and samples were trypsinized and processed as described previously in the legend to Fig. 3. Labeled peptides were visualized by autoradiography. *Panel A*, phosphotryptic peptides from *in vitro* labeled EGFR immunocomplexes from MDA468 cells (4000 cpm). *Panel B*, protein extracts (50 μ g) from MDA468 parental, 5HR, or MDA468c-Src cells which overexpress c-Src, were separated by SDS-PAGE and subjected to immunoblotting with GD11 antibody. *Panel C*, phosphotryptic peptides from *in vitro* labeled, c-Src-associated EGFR from MDA468c-Src cells (4000 cpm).

phosphorylation occur normally when the Y845F receptor is transiently co-expressed in COS cells (38). This finding suggests that a mitogen-activated protein kinase-independent pathway plays a more dominant role in mitogenic signaling emanating from the receptor when it is phosphorylated on Tyr⁸⁴⁵.

That phosphorylation of this Tyr⁸⁴⁵ residue may regulate receptor activity is consistent with the observation that a Tyr⁸⁴⁵ homologue is not found in the EGFR family member erbB3/HER3, which is known to lack kinase activity (44). However, unlike the situation resulting from mutation of the analogous site in other receptor tyrosine kinases, mutation of Tyr⁸⁴⁵ does not appear to alter the EGF receptor's ability to autophosphorylate or to phosphorylate the downstream substrate, Shc (38). In many tyrosine kinases, including Src, JAK 2, and receptors for colony stimulating factor-1, platelet-derived growth factor, insulin, and fibroblast growth factor, the Tyr⁸⁴⁵ homologue is an autophosphorylated residue (35, 36, 45–48). However, to date Tyr⁸⁴⁵ has not been identified as an

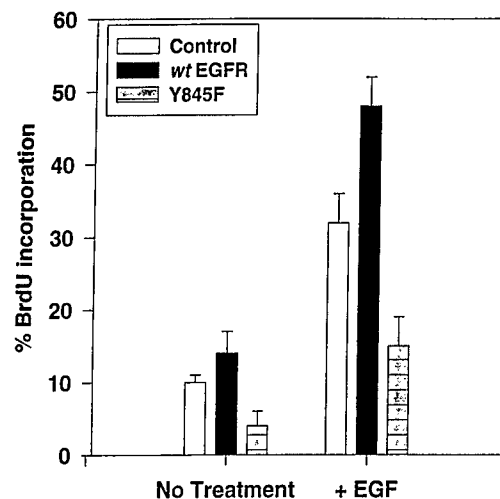


FIG. 8. Phosphorylation of Tyr⁸⁴⁵ is required for EGF-induced DNA synthesis. Neo control cells were transfected with plasmid DNA encoding Y845F or wild type EGFR, cultured for 2 days, serum starved for 30 h, and left untreated or treated with 40 ng/ml EGF for an additional 18 h. Cells were fixed and co-stained for EGFR expression and BrdUrd incorporation. Results are expressed as the mean percent \pm S.E. of cells expressing EGFR that were positive for BrdUrd incorporation. Thirty-five to 75 cells were analyzed for each variable in three independent experiments.

autophosphorylation site for the EGF receptor. This could be due to the highly labile nature of the phosphorylation and/or to the fact that c-Src appears to regulate its phosphorylation (see Figs. 2, 3, and 7). Together these findings raise a number of questions: namely, whether c-Src phosphorylates Tyr⁸⁴⁵ directly, whether binding of c-Src to the receptor causes the receptor to phosphorylate itself, or whether another tyrosine kinase which mediates the phosphorylation is recruited into the complex or activated by c-Src.

Several pieces of evidence support the hypothesis that c-Src phosphorylates the receptor directly. First, Tyr⁸⁴⁵ is homologous to Tyr⁴¹⁶ in Src, which is an autophosphorylation site for Src (39). Additional evidence comes from our studies with both 10T1/2 murine fibroblasts and MDA468 breast cancer cells overexpressing c-Src, where an enhanced phosphorylation of Tyr⁸⁴⁵ is observed. Moreover, other studies from our laboratory demonstrate that overexpression of a kinase inactive form of c-Src in 10T1/2 cells or in MDA468 cells results in a striking decrease in Tyr⁸⁴⁵ phosphorylation (38).⁴ These latter findings indicate that c-Src kinase activity is necessary for the phosphorylation of Tyr⁸⁴⁵ and strongly argue that Tyr⁸⁴⁵ is a direct substrate of c-Src. Last, *in vitro* affinity precipitation and Far Western analyses (Fig. 1, this report, and Refs. 29, 49, and 50) demonstrate that the c-Src SH2 domain can bind activated EGFR specifically and directly, suggesting that recruitment of other tyrosine kinases is not necessary to mediate the phosphorylation of Tyr⁸⁴⁵. However, other EGFR family members (including HER2/neu) (2, 51, 52) and several cytosolic tyrosine kinases, such as other c-Src family members (13) and JAK kinases (53, 54), have been reported to be involved in receptor-mediated signaling, and we cannot exclude their possible involvement in phosphorylation of Tyr⁸⁴⁵ or of Tyr¹¹⁰¹. Whether simple binding of c-Src induces a conformational change in the receptor so that it can autophosphorylate is a much more difficult question to address, a question that minimally awaits identification of the c-Src-binding site.

Other investigators have also described Src-mediated phosphorylations on the EGFR, and Wasilenko *et al.* (24) demon-

⁴ J. S. Biscardi and D. A. Tice, unpublished results.

strated that in NIH3T3 cells co-expressing the transforming oncoprotein v-Src along with EGFR, the receptor contained several novel sites of tyrosine phosphorylation, one of which they postulated might be Tyr⁸⁴⁵ (SPY1). Sato *et al.* (55) provide additional evidence for phosphorylation of Tyr⁸⁴⁵ in A431 cells in a c-Src-dependent fashion, while Stover *et al.* (56) showed that Tyr⁸⁹¹ and Tyr⁹²⁰ were phosphorylated in the c-Src-associated EGFR derived from MCF7 cells. However, neither we nor Sato *et al.* (55) have been able to detect phosphorylation of Tyr⁸⁹¹ or Tyr⁹²⁰, and none of these reports have linked the various phosphorylations to biological changes in receptor activity (e.g. mitogenesis, tumorigenesis). Thus, while there is some discrepancy among the different cell systems, our data and those of others indicate that Tyr⁸⁴⁵ is a major c-Src-dependent phosphorylation site on the EGFR, and that it is associated with increases in receptor function. These findings suggest that multiple tyrosine phosphorylations may be regulated by c-Src.

A potential role for Tyr¹¹⁰¹ is more unclear, as this residue is not conserved among EGFR family members and its phosphorylation level *in vivo* is not as noticeably altered upon c-Src overexpression as is that of Tyr⁸⁴⁵ (see Fig. 3). However, Tyr¹¹⁰¹ may function as a docking site for novel or known signaling proteins, perhaps in an SH2-dependent manner similar to that of the other autophosphorylation sites in the COOH terminus. One of the candidate binding proteins is c-Src itself. In peptide inhibition experiments using synthetic peptides to inhibit the binding between the EGFR and the SH2 domain of c-Src, the SH2 domain of c-Src was shown to bind Tyr⁹⁹² (49, 55) and Tyr¹¹⁰¹ (50) preferentially. Thus, c-Src could bind one of these sites, which could position it to phosphorylate Tyr⁸⁴⁵. In MDA468 breast cancer cells, Tyr⁸⁴⁵ appeared to be the site most affected by c-Src. While the data from the 10T1/2 system suggests that the phosphorylation of both Tyr¹¹⁰¹ and Tyr⁸⁴⁵ is dependent on c-Src, it may be that the phosphorylation of each peptide turns over at different rates in different cell types. Also, the endogenous levels of c-Src in the parental MDA468 cells may be capable of phosphorylating Tyr¹¹⁰¹ to a maximal extent, and no further phosphorylation could result from overexpression. In this regard, overexpression of c-Src may allow for maximal phosphorylation of Tyr⁸⁴⁵ if this phosphorylation turns over at a faster rate, which appears to be the case as the results from Fig. 4 indicate.

Our data show that phosphorylation on Tyr⁸⁴⁵ appears to be critical for EGFR-mediated mitogenesis. Moreover, our results (Figs. 3 and 7) suggest that basal levels of c-Src are able to mediate phosphorylation of Tyr⁸⁴⁵ to some extent, and that this phosphorylation is important to receptor function. In a cell where overexpression and/or activation of c-Src has occurred, as is found in breast cancer, the proper negative regulation of this phosphorylation may be lost, resulting in the increased EGF-dependent signaling and tumorigenicity. We speculate that c-Src and EGFR act synergistically (via phosphorylation of the receptor by c-Src) to induce enhanced signaling in cells which overexpress both these kinases.

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Mechanism of biological synergy between cellular Src and epidermal growth factor receptor

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ABSTRACT Overexpression of both cellular Src (c-Src) and the epidermal growth factor receptor (EGFR) occurs in many of the same human tumors, suggesting that they may functionally interact and contribute to the progression of cancer. Indeed, in murine fibroblasts, overexpression of c-Src has been shown to potentiate the mitogenic and tumorigenic capacity of the overexpressed EGFR. Potentiation correlated with the ability of c-Src to physically associate with the activated EGFR and the appearance of two unique *in vivo* phosphorylations on the receptor (Tyr-845 and Tyr-1101). Using stable cell lines of C3H10T $\frac{1}{2}$ murine fibroblasts that contain kinase-deficient (K-) c-Src and overexpressed wild-type EGFR, we show that the kinase activity of c-Src is required for both the biological synergy with the receptor and the phosphorylations on the receptor, but not for the association of c-Src with the receptor. In transient transfection assays, not only epidermal growth factor but also serum- and lysophosphatidic acid-induced DNA synthesis was ablated in a dominant-negative fashion by a Y845F mutant of the EGFR, indicating that c-Src-induced phosphorylation of Y845 is critical for the mitogenic response to both the EGFR and a G protein-coupled receptor (lysophosphatidic acid receptor). Unexpectedly, the Y845F mutant EGFR was found to retain its full kinase activity and its ability to activate the adapter protein SHC and extracellular signal-regulated kinase ERK2 in response to EGF, demonstrating that the mitogenic pathway involving phosphorylation of Y845 is independent of ERK2-activation. The application of these findings to the development of novel therapeutics for human cancers that overexpress c-Src and EGFR is discussed.

Considerable evidence has accumulated in recent years to suggest that cellular Src (c-Src) and members of the epidermal growth factor (EGF) receptor (EGFR) family are critical elements in the etiology of multiple human cancers. Both kinases are found overexpressed in many of the same types of tumors, including glioblastomas and carcinomas of the colon, breast, and lung (1–4), raising the question of whether they functionally interact to promote the growth of these malignancies. In breast cancer, overexpression of EGFR family members is estimated to occur in 60% or more of the cases (5), and overexpression of the family member HER2/NEU, has been associated with a poor prognosis for the disease (6). Recent reports have also described overexpression of c-Src in a significant majority of patients with breast cancer, a frequency that approaches 100% (1). Studies to assess the oncogenic potential of each kinase have shown that the EGFR is tumorigenic when overexpressed in cultured fibroblasts and activated by ligand (7, 8), but overexpression of c-Src alone is insufficient for malignant transformation (9, 10).

A possible role for c-Src in tumorigenesis was revealed when it was demonstrated in C3H10T $\frac{1}{2}$ murine fibroblasts that

co-overexpression of c-Src and the EGFR resulted in a synergistic increase in EGF-induced DNA synthesis, growth in soft agar, and tumorigenesis, as compared with cells overexpressing either the EGFR or c-Src alone (11). This cooperation correlated with the EGF-dependent formation of a physical complex between c-Src and the EGFR (11), the appearance of two unique sites of tyrosine phosphorylation (Y845 and Y1101) on the c-Src-associated EGFR, and increased phosphorylation of receptor substrates (11). These results suggest that one mechanism by which c-Src could augment the mitogenic/tumorigenic activity of the receptor is by associating with and hyperactivating the receptor by phosphorylation of novel tyrosine residues. Co-overexpression, co-association, and phosphorylation of Y845 and Y1101 have also been observed in human tumor cells (12–15), suggesting that synergism between c-Src and the EGFR may occur in a subset of human tumors as well as in murine fibroblasts.

To determine whether phosphorylation of Y845 or Y1101 is critical to the biological synergy between c-Src and the EGFR and to determine whether c-Src is responsible for mediating the phosphorylations, we analyzed a panel of murine fibroblasts that overexpressed either wild-type (wt) c-Src (K+ c-Src) or kinase-defective c-Src (K- c-Src) alone or together with the EGFR for growth properties and the presence of a stable complex containing the EGFR and c-Src. We found that K- c-Src inhibits EGF-dependent growth in soft agar and tumorigenesis in nude mice even though it is still capable of associating with the receptor. However, K- c-Src was unable to mediate the phosphorylation of Y845 on the receptor. As a direct test of the requirement of this phosphorylation for receptor function, we engineered a variant receptor harboring a Y845F mutation in the EGFR and observed that this mutated receptor ablated EGF, serum, and lysophosphatidic acid (LPA)-induced DNA synthesis without inhibiting receptor kinase activity or activation of the extracellular signal-regulated kinase ERK2. The data support a model wherein phosphorylation of Y845 on the EGFR by c-Src is required for EGF-induced mitogenesis and tumorigenesis in a manner that appears to be independent of ERK2.

MATERIALS AND METHODS

Cell Lines. The derivation, characterization, and maintenance of the clonal C3H10T $\frac{1}{2}$ murine fibroblast cell lines Neo (control), K+ (wt chicken c-Src overexpressors), K- (A430V kinase-deficient, chicken c-Src overexpressors), EGFR (wt human EGFR overexpressors), and EGFR/K+ (wt EGFR/wt c-Src double overexpressors) have been described previously (10, 11, 16). EGFR/K- (wt EGFR overexpressors/kinase-

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: c-Src, cellular Src; EGF, epidermal growth factor; EGFR, EGF receptor; K+, wild-type c-Src; K-, kinase-deficient c-Src; wt, wild-type; LPA, lysophosphatidic acid; BrdUrd, 5-bromodeoxyuridine; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; GPCR, G protein-coupled receptor.

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deficient c-Src) cell lines were derived by infection of K⁻ cells with a recombinant amphotropic retrovirus encoding the human EGFR (8), cloning by limiting dilution, and screening for overexpression of the receptor and maintenance of K⁻ c-Src by Western immunoblotting. Clonal cell lines used in this study were estimated to express 25,000–60,000 human EGFRs per cell, based on comparative Western blotting analysis that used as a standard a C3H10T $\frac{1}{2}$ cell line that by Scatchard analysis was shown to express approximately 200,000 receptors per cell (SHR11 cells) (11). Clones included EGFR₅, EGFR₈, EGFR₂₇, EGFR/K⁺₈, EGFR/K⁺₉, EGFR/K⁺₁₀, and EGFR/K⁻₂, EGFR/K⁻₅, EGFR/K⁻₄₁, and EGFR/K⁻₅₆. K⁺ and K⁻ c-Src overexpression was estimated to be 20- to 25-fold over endogenous.

Western Immunoblotting. Western blot analysis was performed as previously described (11, 16), using Ab-4 rabbit polyclonal antibody (Calbiochem) or F-4 mouse monoclonal antibody (mAb)(Sigma) to detect the EGFR, purified 2–17 mAb (Quality Biologicals; Gaithersburg, MD) or EC10 ascites fluid mAb (prepared in our laboratory and used at a 1:10,000 dilution) to identify c-Src, polyclonal anti-SHC antibody (Upstate Biotechnology; Lake Placid, NY) to visualize SHC, B3B9 mAb (17) to detect MAPK, polyclonal anti-phospho-MAPK antibody (Promega), or 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology) to detect tyrosine-phosphorylated SHC. ¹²⁵I-labeled protein A (ICN) or ¹²⁵I-labeled goat anti-mouse or anti-rabbit Ig (New England Nuclear) and autoradiography were employed to localize binding of primary antibodies.

Colony Formation in Soft Agar and Tumorigenicity. Anchorage-independent growth was measured as previously described (11). Colonies were stained for 20 hr at 37°C in a solution of iodinitrotetrazolium violet (1 µg/ml; Sigma) in water and counted by using EAGLESIGHT analysis software (Stratagene). The soft agar colony data include analysis of three separate clones for each cell type, EGFR₅, EGFR₈, EGFR₂₇, EGFR/K⁺₈, EGFR/K⁺₉, EGFR/K⁺₁₀, EGFR/K⁻₂, EGFR/K⁻₅, and EGFR/K⁻₅₆. Assessment of tumor formation in Taconic *nu/nu* mice was performed as previously described (11).

In Vitro Kinase Assay, Metabolic Labeling with ³²P_i, and Two-Dimensional Tryptic Phosphopeptide Analysis. Methods for immunoprecipitation, *in vitro* kinase assay, metabolic ³²P_i labeling, and two-dimensional phosphopeptide analysis have been described (11, 15). In the metabolic labeling experiments, 5 µM pervanadate and 3 mM H₂O₂ were added to cells simultaneously with 100 ng/ml EGF and incubated for 5 min before harvesting.

5-Bromodeoxyuridine (BrdUrd) Incorporation. A pcDNA3 vector (Invitrogen) encoding human EGFR with a Y845F mutation was constructed by inserting a *Dra*III–*Bst*EII fragment containing the Y845F mutation (from plasmid pCO11, gift of L. Beguinot, Laboratory of Molecular Oncology, Milan, Italy) into the corresponding *Dra*III–*Bst*EII site of pcDNA3 encoding wt EGFR (gift of S. Decker, Parke-Davis, Ann Arbor, MI). K⁺ cells were transiently transfected with 4 µg of vector, wt EGFR, or Y845F EGFR plasmid DNA by using 30 µg of Superfect (Qiagen; Chatsworth, CA) according to manufacturer's directions and incubated in a humidified, 37°C, 5% CO₂/95% air atmosphere for 48 hr to allow a confluent monolayer to form. Transfected cells were then serum-starved for 30 hr prior to addition of 100 µM BrdUrd and 40 ng/ml EGF, 10% fetal bovine serum (FBS) in growth medium, or 10 µM LPA, at which time they were incubated for an additional 18 hr and costained for human EGFR expression and BrdUrd incorporation as described by the manufacturer of the BrdUrd-specific mAb (Boehringer Mannheim). Specifically, fixed cells were treated with 2 M HCl for 1 hr at 37°C and incubated with a mixture of primary antibodies (1:100 dilution of EGFR-specific Ab-4 and a 1:15 dilution of anti-BrdUrd mAb),

followed by incubation with a mixture of secondary antibodies (75 µg/ml fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and 4 µg/ml Texas red-conjugated goat anti-mouse IgG, both from Jackson ImmunoResearch).

Transient Transfections. COS-7 cells were transiently transfected with plasmids encoding SHC and ERK2, by using Superfect as described above. HA-SHC (gift of K. Ravichandran; Univ. of Virginia; HA indicates influenza virus hemagglutinin) or Flag-ERK2 (gift of M. Weber; Univ. of Virginia) was transfected at a 1:5 ratio with or without either wt EGFR or Y845F mutant EGFR and incubated in a humidified, 37°C,

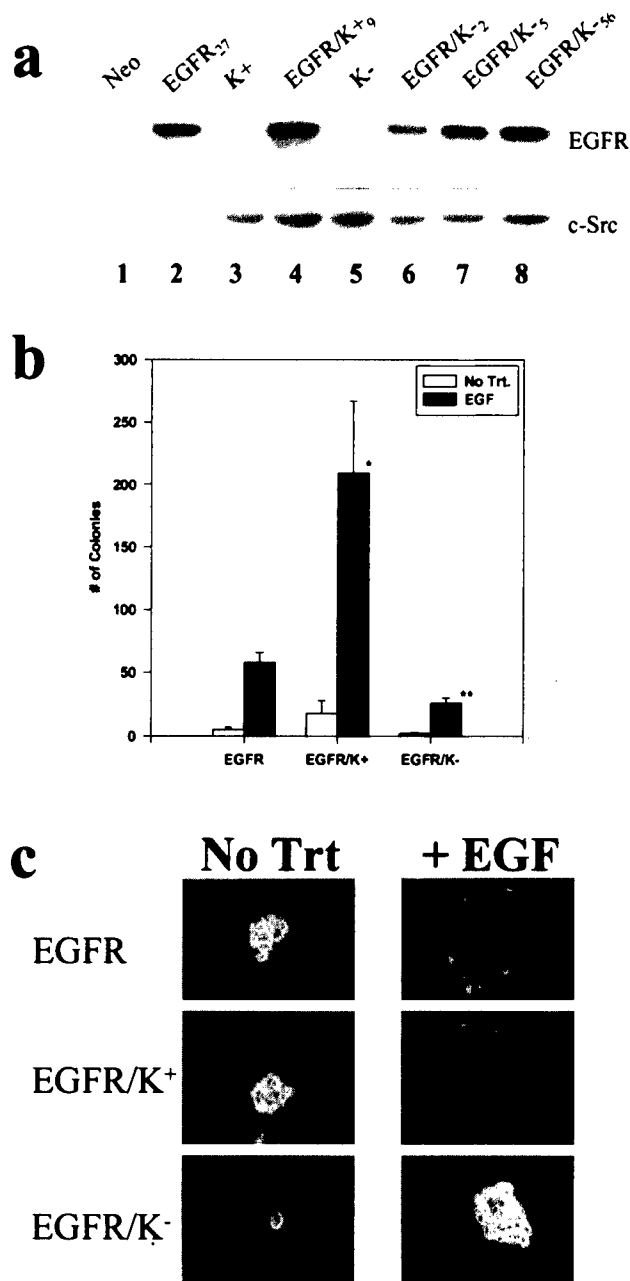


FIG. 1. Dominant repressive effect of K⁻ c-Src on EGF-induced soft agar colony formation. (a) Western immunoblot analysis of C3H10T $\frac{1}{2}$ murine fibroblast clonal cell lines stably overexpressing EGFR and either wt (K⁺) or kinase-deficient (K⁻) c-Src. (b) Values for number of colonies are the mean ± SEM of at least six experiments in which 10⁵ cells of each clone were seeded per plate in triplicate. Three clones of each cell type were averaged. *, *P* < 0.04 and **, *P* < 0.002 compared with EGFR. (c) Photomicrographs of representative fields of soft agar colonies formed from the indicated cell lines were taken after 2 weeks of growth. Trt, treatment. (×200.)

Table 1. K- c-Src completely ablates tumor formation in nude mice in 10T $\frac{1}{2}$ clones

Cell line	Tumor volume,* mm ³
EGFR ₈	295 \pm 124
EGFR/K+ ₉	1,592 \pm 598
EGFR/K-	0†

*Mean tumor volume \pm SEM of eight individual sites was measured at day 52 after subcutaneous injection.

†EGFR/K- represents the mean tumor volume of two individual clones, EGFR/K-₄₁ and EGFR/K-₅₆.

5% CO₂/95% air atmosphere for 48 hr to allow a confluent monolayer to form. Transfected cells were then serum-starved overnight and either stimulated with 100 ng/ml EGF for 10 min or left untreated. Extracts were immunoprecipitated with either 12CA5 anti-HA antibody (Babco: Richmond, CA) or anti-Flag M2 affinity gel (Kodak) and resolved by SDS/PAGE.

RESULTS AND DISCUSSION

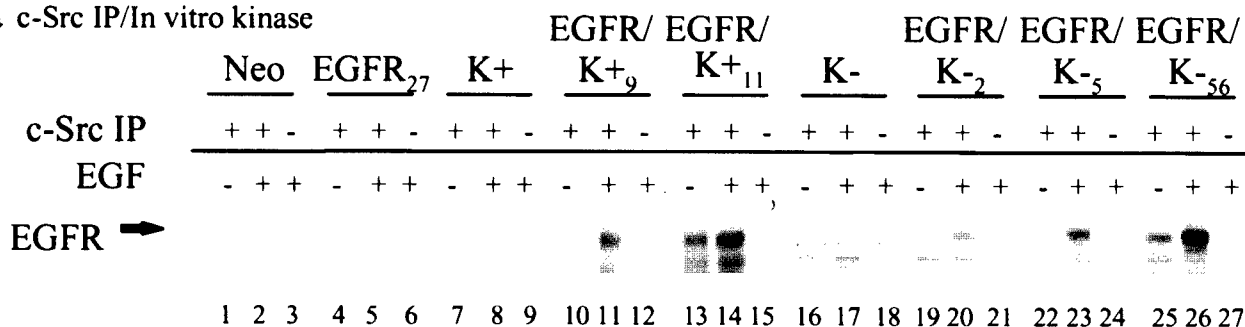
To determine whether phosphorylation of Y845 or Y1101 was dependent on c-Src catalytic activity, clonal C3H10T $\frac{1}{2}$ fibroblasts that stably overexpress wt EGFR and K- chicken c-Src were created from a stable K- c-Src expressing line, as described in *Materials and Methods*. Fig. 1a shows the levels of receptor and c-Src that are expressed in the various cell lines used in this study. EGFR/K- clones (lanes 6–8) expressed levels of receptor comparable to those in EGFR/K+ (lane 4) and EGFR (lane 2) lines, whereas all clones expressing K- c-Src (lanes 5–8) contained amounts of c-Src comparable to those of clones expressing K+ c-Src (lanes 3 and 4). Fig. 1b shows that the EGFR/K- clones exhibited diminished anchorage-independent growth in the presence of EGF compared with EGFR/K+ double overexpressors, demonstrating a requirement for the kinase activity of c-Src for potentiation of EGF-induced soft agar growth. Moreover, relative to cells overexpressing EGFR alone, the EGFR/K- clones also showed reduced soft agar growth, indicating that K- c-Src can function in a dominant-negative fashion for EGFR-induced colony formation. The dominant-negative effect was manifested by both reduced number (Fig. 1b) and significantly smaller average size (Fig. 1c) of the EGFR/K- colonies as compared with those of EGFR/K+ or EGFR cells. As pre-

viously reported, Neo control and K+ c-Src cells produced no or significantly fewer colonies than EGFR cells (11). K- c-Src cells also gave no colonies (data not shown). Table 1 shows that the growth of tumors *in vivo* was completely ablated in mice injected with EGFR/K- cells compared with EGFR or EGFR/K+ cells, demonstrating that K- c-Src has an even stronger dominant-negative effect on tumor growth *in vivo* than on growth in soft agar. Together these results underline the requirement for c-Src kinase activity in both the potentiating effect of overexpressed wt c-Src and the ability of overexpressed EGFR alone to induce oncogenic growth.

To determine whether K- c-Src might be eliciting its biological effects through the receptor, we examined the association between the two kinases, using an immune complex *in vitro* kinase assay as previously described (11). c-Src was immunoprecipitated from the C3H10T $\frac{1}{2}$ clones with a chicken c-Src-specific antibody, EC10, to minimize recognition of endogenous c-Src and to determine whether the exogenously expressed K- c-Src could interact with the EGFR. An EGF-sensitive *in vitro* phosphorylation of an \approx 170-kDa protein was observed in the c-Src immunoprecipitates prepared from EGFR/K- (Fig. 2a, lanes 20, 23, and 26) as well as from EGFR/K+ cells (lanes 11 and 14). These results demonstrate that c-Src kinase activity is not required for association and suggest that K- c-Src may be eliciting its dominant negative effects (at least in part) directly through the receptor, since the association is still intact.

As described before (11), two tryptic phosphopeptides appear in the map of *in vitro* phosphorylated receptor associated with K+ c-Src (Fig. 3B) that are either absent or present in reduced amounts in the map of "free" activated receptor (Fig. 3A). These peptides contain Y845 and Y1101, whose identification is described in ref. 15. In contrast to the receptor associated with K+ c-Src, phosphorylation of Y845 was undetectable in receptor associated with K- c-Src (Fig. 3C and D), while the level of Y1101 phosphorylation was visible but reduced. Similar results were observed in ³²P_i metabolic labeling experiments (Fig. 3E and F). Phosphorylation on Y845 was also observed in cells expressing endogenous levels of c-Src, but only after treatment with pervanadate (15), suggesting that this site is phosphorylated in the absence of overexpression of c-Src and that it is rapidly turned over. These results indicate that phosphorylation of Y845, and to a lesser

a c-Src IP/In vitro kinase



b c-Src IP/c-Src Blot



FIG. 2. K- c-Src associates with the EGFR. Confluent, serum-starved cells were untreated or treated with EGF (100 ng/ml) for 15 min, and extracts were immunoprecipitated with either chicken c-Src-specific mAb EC10 (designated +) or a negative control mouse IgG (-). (a) Immunocomplexes were subjected to an *in vitro* kinase assay using [γ -³²P]ATP, and phosphorylated products were analyzed by SDS/PAGE and autoradiography. The region of the autoradiograph around the 170-kDa products is shown. (b) The amount of c-Src in each precipitate was visualized by Western immunoblot analysis with EC10 mAb. K- c-Src in EGFR/K- clones was verified to be catalytically inactive in the immune complex kinase assay (data not shown).

extent of Y1101, depends on the kinase activity of c-Src, both *in vitro* and *in vivo*.

The position corresponding to Y845 is highly conserved among serine/threonine and tyrosine kinases and is situated in the activation loop between subdomains VII and VIII (18). Three-dimensional structural studies of several kinases have pointed to the importance of phosphorylation of this residue in stabilizing the activation loop in a conformation favorable for substrate and ATP binding (19–21). In agreement with the structural data, mutational analysis of the corresponding residue in tyrosine kinase receptors, including p185^{neu}, a highly conserved family member, has shown a requirement for phosphorylation of this residue for full biological function in response to ligand (22–27). Y845 homologues in other tyrosine kinase receptors have all been shown to be autophosphorylation sites. In contrast, Y845 of the EGFR has not been identified as such, and its importance to EGFR function has not been ascertained. The failure to identify Y845 as a site of autophosphorylation may reflect either the highly labile nature of the phosphorylation or the c-Src dependency of the phosphorylation (15, 28).

To determine whether phosphorylation of Y845 is required for receptor kinase activity, we compared wt EGFR autokinase activity with that of a mutant Y845F EGFR. Similar amounts of autophosphorylation were observed in an *in vitro* kinase assay of Y845F or wt EGFR immunoprecipitated from transiently transfected and EGF-stimulated COS-7 cells (Fig. 4). Further evidence that the EGFR, unphosphorylated on

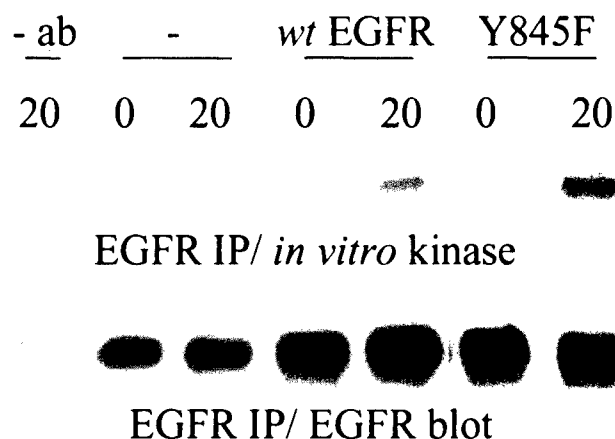


FIG. 4. Phosphorylation of Y845 is not essential for EGFR autokinase activity. COS-7 cells were transiently transfected with either vector alone (–), or plasmids encoding Y845F or wt EGFR and stimulated with EGF for 5 min. The EGFR was immunoprecipitated from extracts and subjected to an *in vitro* kinase assay for 0 or 20 min. The reaction was stopped with the addition of sample buffer, and the products were resolved by SDS/PAGE and transferred to a membrane. After autoradiography, EGFR was detected by Western immunoblotting and visualized by using enhanced chemiluminescence (ECL; Amersham).

Y845F, retains its ability to autophosphorylate is provided by a comparison of the tryptic phosphopeptide maps of wt EGFR from EGFR cells (Fig. 3A) and wt EGFR from EGFR/K– cells

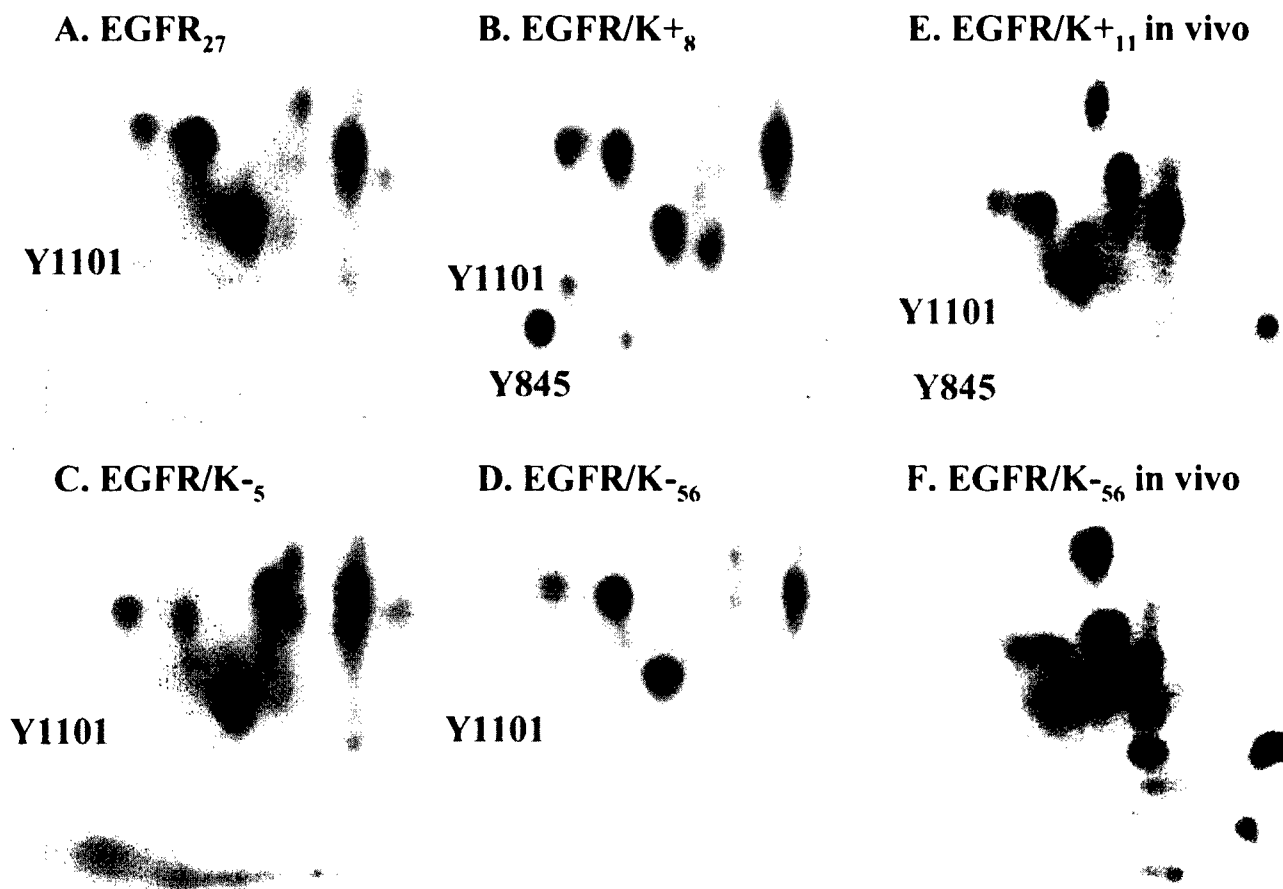


FIG. 3. Y845 is not phosphorylated in EGFR complexed with K– c-Src. (A–D) The 170-kDa bands that were phosphorylated *in vitro* (as in Fig. 2) in c-Src (B–D) or receptor (A) immunocomplexes prepared from the indicated cell lines were excised, digested with trypsin, resolved by two-dimensional electrophoresis/chromatography, and subjected to autoradiography. The positions of peptides containing Y845 and Y1101, which were identified previously (15), are indicated. (E and F) Receptor immunoprecipitates from the indicated cell lines that had been metabolically labeled with ³²P_i were analyzed as in A–D. Equal cpm were loaded in A–D and in E vs. F. The apparent increase in tyrosine phosphorylation in F is due to a slightly darker exposure compared with E to emphasize the complete ablation of Y845 phosphorylation. The appearance of darker or novel spots in F was not reproduced in repeated experiments.

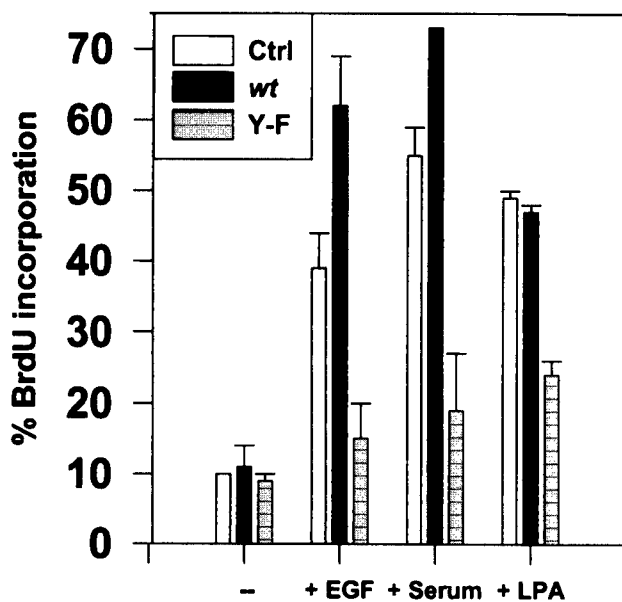


FIG. 5. Phosphorylation of Y845 is essential for EGFR function. K+ cells were transfected with plasmid DNA encoding Y845F (Y-F) or wt EGFR, cultured for 2 days, serum starved for 30 hr, and left untreated or treated with 40 ng/ml EGF, 10% fetal bovine serum, or 10 μ M LPA for 18 hr. Results are expressed as the mean percent \pm SEM of cells expressing EGFR that were positive for BrdUrd incorporation. Thirty-five to 100 cells were analyzed for each variable in three independent experiments.

(Fig. 3 C and D), where in the latter instance, autophosphorylation is maintained in the absence of detectable Y845 phosphorylation. These data suggest that, unlike other receptor tyrosine kinases mutated at the Y845 homologue, the Y845 mutant EGFR maintains its ability to autophosphorylate.

To test whether phosphorylation on Y845 is important for the mitogenic function of the EGFR independent of its autokinase activity, we transiently transfected a Y845F mutant or wt receptor into K+ cells and assessed mitogenesis by measuring EGF-induced BrdUrd incorporation into newly synthesized DNA. In contrast to the wt receptor, the Y845F mutant was unable to stimulate DNA synthesis upon EGF treatment (Fig. 5). Indeed, the reduced level of BrdUrd incorporation, which approached that of serum-starved cells, indicated that Y845F EGFR is capable of interfering with signaling through endogenous receptors, thereby acting in a dominant-negative fashion. These data support the hypothesis that phosphorylation of Y845 is required for the EGF-induced mitogenic function of the receptor.

Surprisingly, the Y845F variant of the EGFR also inhibited serum-induced DNA synthesis in a dominant-negative manner (Fig. 5). The mechanism of this inhibition is unclear at the present time. However, the EGFR has recently been shown to play an essential role in signaling and growth stimulation through G protein-coupled receptors (GPCR) (29), and the Src family of tyrosine kinases has also been directly implicated in GPCR-mediated mitogen-activated protein kinase (MAPK) activation (30, 31). c-Src is thought to be responsible for phosphorylating the EGFR in response to GPCR activation (32), leading to the generation of docking sites. The major mitogenic component of serum is LPA, a ligand for GPCR (33). Therefore, one possible mechanism by which the Y845F EGFR could prevent serum-induced BrdUrd incorporation might be the inability to phosphorylate Y845 via a GPCR route. Indeed, Y845F mutant EGFR was able to reduce (but not ablate) induction of DNA synthesis by LPA, demonstrating an involvement of EGFR signaling in the GPCR pathway (Fig. 5).

Because the EGFR is known to signal to MAPK via a SHC-Grb2-SOS-Ras pathway upon both EGF and G protein stimulation, we also tested the ability of the mutant EGFR to

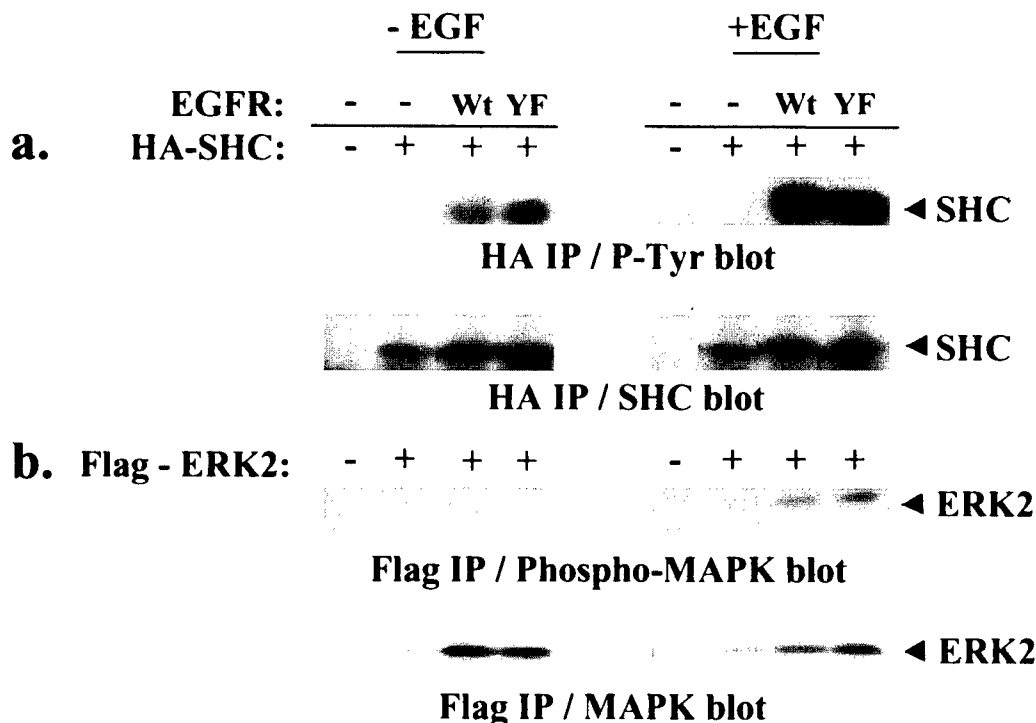


FIG. 6. Y845F mutant receptor retains its ability to phosphorylate SHC and activate MAPK. COS-7 cells were transfected with plasmid DNA encoding HA-SHC or Flag-ERK2 and either Y845F or wt EGFR, cultured for 2 days, serum starved overnight, and left untreated or treated with 100 ng/ml EGF for 10 min. Extracts were immunoprecipitated with 12CA5 anti-HA antibody or anti-Flag M2 affinity gel and resolved by SDS/PAGE. The amount of tyrosine phosphorylated HA-SHC (a) and Flag-ERK2 (b) was observed by Western immunoblot analysis. The amount of wt EGFR or Y845F EGFR expressed in the populations of cells was the same as that shown for Fig. 4.

phosphorylate the direct substrate SHC and to activate ERK2. The presence of the Y845F mutation in the EGFR did not alter the EGF-induced increase in tyrosine phosphorylation of cotransfected SHC or ERK2, compared with wt EGFR (Fig. 6). Minor differences in the relative phosphorylations of either SHC or ERK2 between mutant and wt EGFR were not significant when multiple experiments were quantitated (data not shown). These results suggest that the EGFR stimulates mitogenesis through an ERK2-independent pathway.

The data presented here provide a mechanism for c-Src's role in EGF- and GPCR-mediated DNA synthesis and tumorigenesis. We propose that phosphorylation of Y845 on the EGFR by a c-Src-mediated event is required for EGF- and LPA-induced DNA synthesis. On the basis of the findings that Y845 is not phosphorylated by the wt receptor alone (Fig. 3) and that the kinase activity of c-Src is required for phosphorylation of Y845, we conclude that c-Src is the most likely kinase to phosphorylate the receptor. Interruption of this phosphorylation by overexpressing a kinase-deficient c-Src or a Y845F mutant of the EGFR blocks signaling and thus growth. Furthermore, the block of DNA synthesis by Y845F mutant EGFR is not dependent on its ability to autophosphorylate or to signal to MAPK, suggesting a mechanism of activation in which the activation loop tyrosine does not need to be phosphorylated for kinase activity, but is required for stimulation of a mitogenic pathway not involving ERK2.

These findings have direct implications for the etiology of human cancers. In tumor cells that overexpress both c-Src and the EGF receptor, we postulate that the probability of Y845 phosphorylation increases, an event that results in promotion of growth and anchorage independence. Since phosphorylation of Y845 has been shown to occur in cultured human tumor cells that overexpress c-Src (15), the above paradigm may have relevance for the disease *in situ*. Development of methods to inhibit the ability of c-Src to phosphorylate Y845 may result in a novel, more "tumor-specific" treatment for cancers such as carcinomas of the colon, breast, and lung.

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Review

Tyrosine kinase signalling in breast cancer Epidermal growth factor receptor and c-Src interactions in breast cancer

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Abstract

Both the non-receptor tyrosine kinase, c-Src, and members of the epidermal growth factor (EGF) receptor family are overexpressed in high percentages of human breast cancers. Because these molecules are plasma membrane-associated and involved in mitogenesis, it has been speculated that they function in concert with one another to promote breast cancer development and progression. Evidence to date supports a model wherein c-Src potentiates the survival, proliferation and tumorigenesis of EGF receptor family members, in part by associating with them. Phosphorylation of the EGF receptor by c-SRC is also critical for mitogenic signaling initiated by the EGF receptor itself, as well as by several G-protein coupled receptors (GPCRs), a cytokine receptor, and the estrogen receptor. Thus, c-Src appears to have pleiotropic effects on cancer cells by modulating the action of multiple growth-promoting receptors.

Keywords: c-Src, epidermal growth factor receptor, human epidermal growth factor receptor 2/neu, signal transducers and activators of transcription, tyrosine phosphorylation

Introduction

Recent evidence has implicated an involvement of tyrosine kinases in human breast cancer development. Two families in particular have been examined, namely, the human epidermal growth factor and the Src families of tyrosine kinases.

Human epidermal growth factor receptor (HER1) in human breast cancer

The human epidermal growth factor receptor (HER1) is the prototype of a family that consists of four known members (EGF receptor/HER1, neu/erbB2/HER2, erbB3/HER3, and erbB4/HER4). These receptor tyrosine kinases are characterized by an extracellular ligand-binding domain, an

internal kinase domain, and a carboxyl-terminal domain that contains multiple tyrosine residues. Upon binding of EGF, HER1 dimerizes and becomes phosphorylated on these carboxyl-terminal tyrosyl residues, which in turn act as docking sites for multiple signaling proteins that contain SH2 domains. HER1 plays a variety of roles in normal development, and is found in ductal epithelial cells of normal breast tissue [1**].

The link between HER1 and human cancer initially came from studies by Velu *et al* [2], who demonstrated that cells that overexpress HER1 become transformed when they are grown in the continuous presence of EGF. HER1 is overexpressed in a variety of human cancers, including

EGF = epidermal growth factor; ER = estrogen receptor; ERK = extracellular-signal-regulated kinase; GPCRs = G-protein-coupled receptors; HER = human epidermal growth factor receptor; JAK = janus kinase; LPA = lysophosphatidic acid; MAPK = mitogen-activated protein kinase; STAT = signal transducer and activator of transcription; PI-3K = phosphatidylinositol-3 kinase.

benign skin hyperplasia, glioblastoma and cancers of the breast, prostate, ovary, liver, bladder, esophagus, larynx, stomach, colon, and lung [3*]. Approximately 30% of human breast tumors overexpress HER1, and this overexpression is correlated with a loss of estrogen responsiveness and a poorer prognosis [4,5]. Much evidence suggests that HER1 is involved in later stages of human breast cancer and may play a role in the metastatic process [6].

Human epidermal growth factor receptor 2 (HER2) in breast cancer

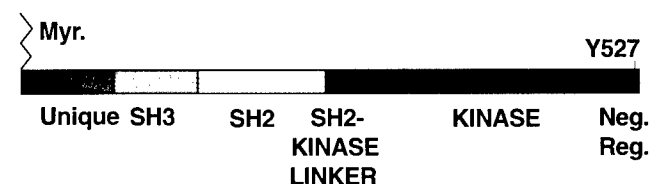
Among the HER family members, HER2 is most closely related to HER1 [7] and has been found to be amplified in 10–35% of human breast carcinomas, an event that portends a poor disease prognosis [8**,9]. Overexpression of HER2 occurs more frequently in the early stages of breast cancer, and is therefore thought to be involved in tumor initiation and early stages of progression [10**]. The involvement of HER2 in human breast cancer is further supported by the success of recent immunotherapy trials, which targeted the receptor in conjunction with current chemotherapeutic protocols [11].

c-Src in breast cancer

c-Src, a nonreceptor tyrosine kinase that is localized to intracellular membranes of the cell, has also been found to be overexpressed or highly activated in a number of human neoplasms, including carcinomas of the breast, lung, colon, esophagus, skin, parotid, cervix, and gastric tissues, as well as in neuroblastomas and myeloproliferative disorders. In several studies that together examined over 125 human breast tumor specimens and cell lines [12,13,14*], more than 70% of the samples contained levels of c-Src tyrosine kinase activity that were two-fold to 50-fold greater than those found in normal breast epithelium or immortalized mammary epithelial cells. This elevated activity could be accounted for solely by an increase in c-Src protein levels, and did not reflect an increase in specific activity of the enzyme [14*]. Such striking increases in c-Src protein levels in a surprisingly high percentage of human breast neoplasias provide correlative evidence that c-Src is involved in some facet of breast cancer development.

Unlike the EGF receptor, overexpression of c-Src alone is insufficient to transform murine fibroblasts in culture or to sustain tumor growth in intact animals [15,16,17**]. However, expression of dominant interfering forms of c-Src (Fig. 1) in cultured murine fibroblasts has shown that c-Src is required for EGF-induced mitogenesis [18*,19], and studies in transgenic mice have demonstrated that c-Src is necessary for induction of mammary tumors by the polyomavirus middle T oncogene [20]. These findings suggest that c-Src may function to promote growth of tumor cells by participating in or augmenting mitogenic signaling pathways that are initiated by extracellular

Figure 1



Structure of c-Src. C-Src is the prototype of a large family of cytoplasmic tyrosine kinases that associate with cellular membranes through lipid modifications at their amino-termini. As a linear molecule, the relationship between the various domains can be seen: an amino-terminal membrane-association domain that contains the site of myristylation; a unique domain that exhibits the widest sequence divergence among family members of any of the domains; an SH3 domain that binds polyproline motifs on target molecules; a SH2 domain that binds phosphotyrosine residues on target molecules; a SH2/kinase linker; the catalytic domain; and the negative regulatory domain that contains the predominant site of tyrosine phosphorylation on the inactive molecule (Y527 in chicken, Y530 in human). Mutations that abrogate myristylation, the SH2 domain, and the catalytic activity were shown to reduce EGF-stimulated DNA synthesis in C3H10T $\frac{1}{2}$ murine fibroblasts, providing evidence for the involvement of c-Src in mitogenic pathways [18*].

growth factors or intracellular oncogenes. There appear to be two prominent roles of c-Src in this regard. One is to modulate receptor function by augmenting signals immediately downstream of the receptors and by regulating endocytosis [1**,21**,22**], and the other is to affect morphogenetic remodeling of the cell by phosphorylating proteins that associate with the actin cytoskeleton [23,24*].

Synergism between c-Src and human epidermal growth factor receptor 1 in oncogenesis

The fact that c-Src and HER1 are co-overexpressed in many of the same tumor types suggests that these two kinases may participate in regulating the genesis and/or progression of human cancers. In a direct test to resolve this question, dual overexpression of both c-Src and HER1 in C3H10T $\frac{1}{2}$ mouse fibroblasts was found to lead to synergistic increases in EGF-induced DNA synthesis, soft agar colony formation, and tumor formation in nude mice, when compared with cells that express only one of the pair [17**]. This enhanced oncogenesis correlated with the EGF-dependent physical association between c-Src and HER1, increased phosphorylation of the HER1 substrates, Shc and phospholipase C γ , and the phosphorylation of two novel tyrosyl residues on the receptor, which have been identified by phosphotryptic mapping to be Tyr 845 and Tyr 1101 [17**,24*]. Stover *et al* [25] showed that activated Src can phosphorylate HER1 at Tyr 891 and Tyr 920 *in vitro*, and that these sites can mediate binding of the SH2 domains of phosphatidylinositol-3 kinase (PI-3K) and Src itself. These same sites have been

found to be phosphorylated in several colorectal carcinomas and MCF7 breast cancer cells, from which c-Src and PI-3K can be coimmunoprecipitated with HER1. These findings support the idea that bidirectional interactions between c-Src and HER1 occur.

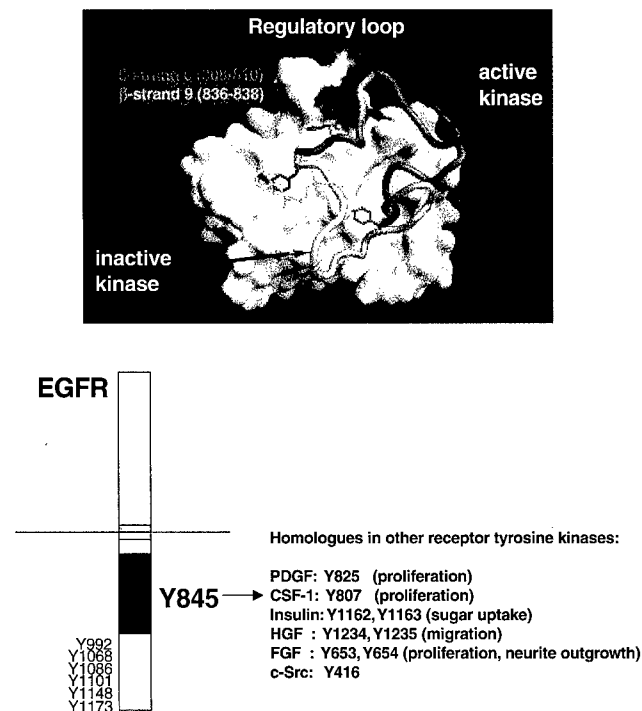
Interestingly, co-overexpression of both HER1 and c-Src also occurs in a subset of human breast cancer cell lines and breast tumor tissues [14^{*}]. Like the mouse 10T $\frac{1}{2}$ fibroblasts, breast tumor cell lines that co-overexpress c-Src and HER1 display EGF-induced complex formation between c-Src and HER1, the appearance of Tyr 845 and Tyr 1101 phosphorylations on the c-Src-associated receptor, and increased phosphorylation of the HER1 effectors Shc and mitogen-activated protein kinase (MAPK), as well as increased tumor size in nude mice when compared with the majority of cell lines that do not overexpress these tyrosine kinases or express only one of the pair. Enhanced MAPK and MEK activity have also been found in human breast tumors that overexpress both c-Src and HER1 [26,27]. These results suggest that c-Src and HER1 act synergistically, and that this interaction is manifested by increased signaling through HER1, unregulated growth, and tumorigenesis. Because complex formation between HER1 and c-Src can be detected only under conditions of mutual overexpression, as is seen in many breast tumors and cell lines, disruption of this complex could provide the basis for novel therapeutic approaches.

Molecular mechanisms of c-Src/human epidermal growth factor receptor 1 synergism

Although little is known about the biologic significance of Tyr 1101 phosphorylation, Tyr 845 is located in the activation loop of the catalytic domain and is particularly intriguing, because its homologs are found in a variety of receptor and nonreceptor tyrosine kinases (Fig. 2) [28]. In fact, substitution of Phe for the Tyr 845 homologs in other receptors renders these receptors catalytically impaired and defective in downstream signaling [29–31]. In contrast, Tyr 845 in the EGF receptor has never been identified as an autophosphorylation site. Failure to detect such a phosphorylation was interpreted to mean either that the site was not phosphorylated or that its phosphorylation was extremely short-lived.

Recently, it was shown [32^{**}] that phosphorylation of Tyr 845 is dependent upon the catalytic activity of c-Src, suggesting that c-Src directly phosphorylates this site. Cells expressing kinase-inactive c-Src not only fail to support phosphorylation of Tyr 845, but also display a drastically decreased ability to grow in soft agar in the presence of EGF and to form tumors in nude mice, suggesting that phosphorylation of Tyr 845 may be critical for mitogenesis and transformation. Indeed, in 10T $\frac{1}{2}$ cells that express either increased or endogenous levels of c-Src, expression of a Tyr845Phe mutant form of HER1 results in a

Figure 2



Tyr 845 is located in the catalytic domain of HER1 in a highly conserved subdomain that has functional homologs in other tyrosine kinases. Tyr 845 resides in the activation loop of the HER1 tyrosine kinase catalytic domain (upper panel), a region that shares a high degree of homology among all tyrosine kinases (lower panel). Upon ligand-binding to the EGF receptor (EGFR), the activation loop containing phosphorylated Tyr 845 is modeled (upper panel [63]) to flip into a configuration that promotes access to ATP and substrate, as is the case with phosphorylation of the analogous tyrosines within other tyrosine kinases. Mutation of sites in other tyrosine kinases that are analogous to Tyr 845 renders the molecules catalytically inactive and abrogates downstream biologic signaling. Similar effects of mutagenizing Tyr 845 have been observed [24^{*},32^{**}]. A unique characteristic of Tyr 845 appears to be that its phosphorylation is mediated by c-Src, whereas homologs in other receptor tyrosine kinases are phosphorylated by the receptors themselves. The phosphorylation of Tyr 845 by c-Src is proposed to facilitate cross-talk between HER1 and signaling pathways activated by other cellular receptors, such as G-protein-coupled receptors and estrogen receptor.

reduction in EGF-, serum-, and lysophosphatidic acid (LPA)-induced DNA synthesis [24^{*},32^{**}]. Thus, c-Src-mediated phosphorylation of Tyr 845 appears to be necessary for the mitogenesis that emanates from HER1.

The finding that serum- and LPA-induced DNA synthesis are affected by the Tyr845Phe mutation suggests that other cell-surface receptors may mediate their effects in part through the EGF receptor. LPA, a major mitogen in serum, is known to activate a G γ -coupled receptor [33]. Several laboratories have recently reported that activation of certain GPCRs can trigger phosphorylation of the EGF

receptor, as well as activation of its downstream effectors Shc and MAPK, and that this activation is dependent on c-Src kinase activity [34*,35,36]. In addition, stimulation of the growth hormone cytokine receptor has been found to induce EGF receptor phosphorylation via janus kinase 2 [37]. Recent work in our laboratory (Biscardi *et al*, unpublished data) has demonstrated that treatment of 10T $\frac{1}{2}$ cells with different GPCR ligands (thrombin, endothelin, and LPA) or with growth hormone induces increases in overall tyrosine phosphorylation of HER1, as well as in Tyr 845 phosphorylation. Interestingly, the kinase activity of c-Src is also required for phosphorylation of Tyr 845 via these alternate receptors, and mitogenesis is dependent on the phosphorylation of Tyr 845. 10T $\frac{1}{2}$ cells that express the Tyr845Phe variant of HER1 are impaired in their ability to synthesize DNA in response to these stimuli. However, the weakly mitogenic effects of isoproterenol, which signals through a Gs α -coupled pathway, are not affected by the Tyr845Phe mutation, indicating that this mutation does not act as a general inhibitor of mitogenesis.

Estrogen receptor, c-Src, and human epidermal growth factor receptor 1

Accumulating evidence also points to an intricate network of cross-talk between the estrogen receptor and HER1. Early work by Ignar-Trowbridge *et al* [38,39] demonstrated that EGF can transcriptionally activate genes that contain estrogen response elements. More recently, Migliaccio *et al* [40,41] showed that estrogen is able to activate many of the effectors classically thought to be linked to the EGF receptor signaling pathway, including c-Src, Ras, and MAPK. These researchers have also shown that estrogen requires c-Src kinase activity in order to trigger its mitogenic effects [42*]. To further investigate this phenomenon, our laboratory has examined the effects of the Tyr845Phe mutation on estrogen-dependent DNA synthesis in the estrogen-responsive MCF7 breast cancer cell line. As was the case for the GPCR and cytokine receptor coupled agonists, estrogen-stimulated DNA synthesis was decreased to basal levels as a result of expression of the Y845F mutant (Biscardi *et al*, unpublished data). Taken together, these findings suggest the possibility that the EGF receptor plays an important, perhaps widespread, role in mediating the cell's response to an array of external signals and that the c-Src mediated phosphorylation of EGF receptor Tyr 845 appears to be a critical event in this process (Fig. 3).

Human epidermal growth factor receptor 2 and c-Src in breast cancer

Evidence supporting bidirectional interactions between c-Src and the EGF receptor raises the question of whether c-Src interacts in a similar manner with other HER family members. Some indications that HER2 and c-Src can physically and/or functionally interact have emerged over recent years, but little is currently known about the relationship between c-Src and either HER3 or HER4. *In*

vitro studies have also demonstrated that HER2/*neu* can associate with the SH2 domain of c-Src in a tyrosine phosphorylation-dependent manner [43,44**], and *in vivo* coassociation between HER2/*neu* and c-Src has been detected in murine mammary tumors, human breast cancer cell lines, and human tumor tissues [44**] (Belsches-Jablonski AP *et al*. unpublished data). Furthermore, transgenic murine tumor tissues or human mammary epithelial cell lines expressing mutationally activated *Neu* exhibit a correlative increase in c-Src activity [44**,45]. These results suggest that c-Src may be downstream of HER2 signaling. It has also been demonstrated *in vitro* [25], however, that c-Src is able to phosphorylate HER2 at nonautophosphorylation sites. The identity of these sites, their existence in intact cells, and their functional significance have not yet been determined. Nevertheless, the currently available information suggests that HER2 and c-Src are able to interact physically and that bidirectional signaling may be a mechanism of interaction between these two tyrosine kinases, as it is for HER1 and c-Src.

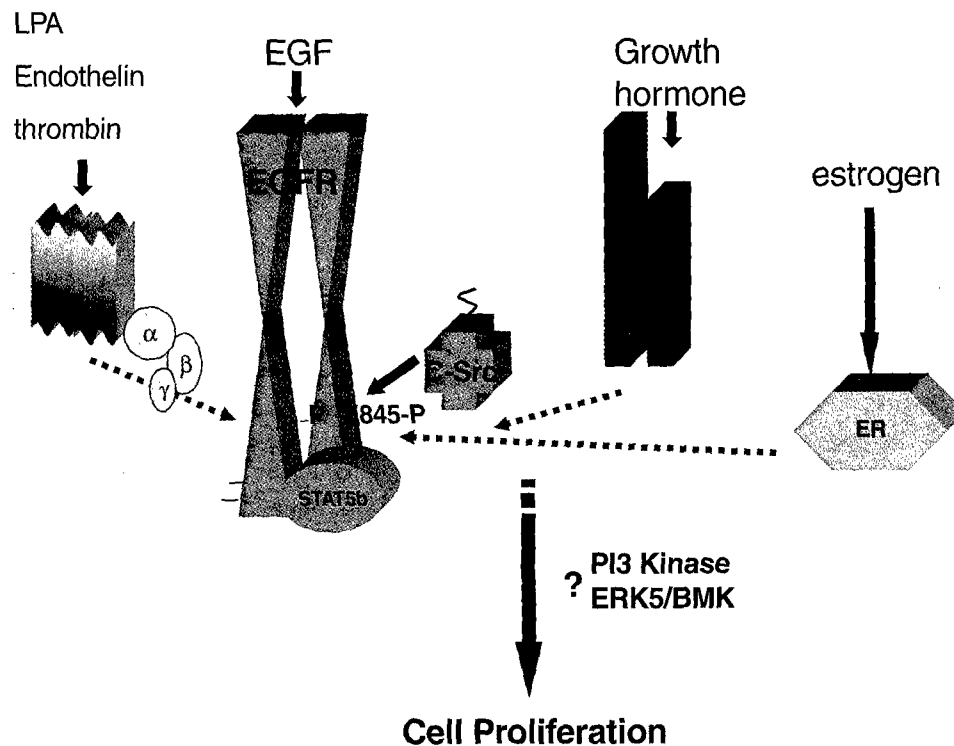
The functional consequences of coassociation between HER2 and c-Src remain unclear. In fact, available evidence suggests that the HER2-c-Src interaction may affect different parameters of oncogenesis to different extents and perhaps by different mechanisms than the HER1-c-Src association. Results from recent studies of MCF10A cells that ectopically express mutationally activated rat p185^{neu} [45], and a panel of 13 human breast cancer cell lines and 13 human mammary tumor samples (Belsches-Jablonski AP *et al*. unpublished data) suggest that the HER2-c-Src complex may play an important role in heregulin-stimulated anchorage-independent growth and antiapoptotic or survival mechanisms, but have less of an effect on anchorage-dependent growth. In contrast, the HER1-c-Src complex has been found to have striking effects on anchorage-dependent growth and on anchorage-independent growth, but its role in survival signaling is unclear.

Whether c-Src mediates the phosphorylation of the Tyr 845 homolog in HER2 (Tyr 877) as it does in HER1 is not known. The comparable site in the activated, rat p185^{neu} protein (Tyr 882) is an autophosphorylation site, and mutation of this residue reduces the intrinsic kinase activity of the protein and its transforming potential [46*]. These findings suggest that the Tyr 845 homolog in p185^{neu} or HER2 functions in a manner more similar to the majority of tyrosine kinase receptors than it does to HER1, and raise the possibility that the mechanism by which c-Src interacts with HER2 may be distinct from that by which c-Src interacts with HER1.

Signals activated by c-Src/human epidermal growth factor receptor 1 interactions

Although mutation of Tyr 845 has profound effects on the cell's ability to respond mitogenically to EGF, many of the

Figure 3



HER1 acts as a central mediator for multiple signaling pathways. A variety of extracellular ligands trigger the phosphorylation of HER1 on Tyr 845. These include the following: thrombin, endothelin, and LPA, which bind G-protein coupled receptors; growth hormone, which binds a cytokine receptor; and estrogen, which binds a steroid hormone receptor. Moreover, c-Src kinase activity is required for the ability of LPA, endothelin, growth hormone, and estrogen to induce phosphorylation of Tyr 845. We hypothesize that c-Src-mediated phosphorylation of Tyr 845 is a central signaling event and is required for mitogenesis to occur in response to a variety of external stimuli in addition to EGF. The signaling molecules that transmit mitogenic cues from phosphorylated Tyr 845 have yet to be delineated, but may include such effectors as STAT5b, PI-3K, or ERK5. ER, estrogen receptor.

downstream targets of HER1 are unaffected. The Y845F mutant HER1 kinase activity appears to be unchanged, as does its ability to associate with c-Src. Moreover, the phosphorylation and/or activation of a number of HER1 effectors, including Shc, MAPK, signal transducer and activator of transcription (STAT)3, and phospholipase Cy [32**] (Tice and Biscardi, unpublished data) are likewise unaffected. However, recent evidence, produced in a collaborative effort between our laboratory and that of Silva (unpublished data), suggests that STAT5b might be a physiologically relevant downstream effector of Tyr845.

The STATs are a family of transcription factors that are activated at the plasma membrane by tyrosine phosphorylation in response to signals from cytokine and growth factor receptors [47*,48]. Tyrosine phosphorylation results in STAT dimerization, nuclear translocation, and binding of STAT dimers to consensus elements upstream of regulated genes.

Increasing evidence indicates that STAT proteins are involved in the process of oncogenesis [49*,50]. Two lab-

oratories have shown that STAT3 is required for v-Src transformation [51**,52**], whereas deGroot *et al* [53] demonstrated that active STAT5 is necessary for the soft agar growth of BCR-Abl transformed leukemia cells. Recent studies [54**] have also indicated a direct role for c-Src in the activation of STAT proteins. For example, c-Src was shown to mediate the EGF stimulation of STATs 1, 3, 5a, and 5b in NIH3T3 cells engineered to overexpress HER1, as well as in A431 cells, which endogenously express high levels of HER1. In contrast, another group [55] has recently described a role for c-Src in the tyrosine phosphorylation (but not the transcriptional activation) of STAT5a and STAT5b in a COS cell transfection model.

Our recent studies (Silva *et al*, unpublished data) indicate a role for the STAT proteins in signaling pathways that are activated in 10T $\frac{1}{2}$ and breast cancer cells co-overexpressing c-Src and EGF receptor. We have shown that c-Src tyrosine kinase activity is required for maximal transcriptional activation of STAT5b by EGF, and that phos-

phorylation of Tyr 845 is required for both the EGF-induced association between STAT5b and HER1 as well as tyrosine phosphorylation of STAT5b. These studies suggest a model whereby HER1 and c-Src overexpression and EGF stimulation lead to the phosphorylation of Tyr845 and the recruitment and activation of STAT5b.

A number of other signaling molecules have also been linked to EGF-induced mitogenesis in various cell systems, and should be considered as additional candidates for downstream effectors of Tyr845. These signaling molecules include PI-3K, big MAPK [BMK1 or extracellular-signal-regulated kinase (ERK)5], and the transcription factor Myc. After growth factor activation, PI-3K interacts, through its SH2 domain, with tyrosine phosphorylated growth factor receptors, resulting in an increase in PI-3K activity. Studies using specific antibodies to PI-3K [56*] demonstrated that its catalytic activity is required for EGF (and platelet-derived growth factor)-induced mitogenesis. Although PI-3K associates with the EGF receptor, the binding site has not been characterized and thus it is interesting to speculate that this function may be fulfilled by Tyr 845. ERK5, a member of the MAPK family, was first shown to be activated in response to oxidative stress, hyperosmolality, and serum. Recent studies [57**,58] have shown that this kinase is also activated in response to EGF and nerve growth factor. Furthermore, dominant-negative ERK5 blocks EGF-induced cell proliferation in a breast epithelial cell line by preventing cells from entering the S phase [57**]. Studies in mouse fibroblasts have shown that c-Src kinase is required for ERK5 activation in response to hydrogen peroxide. Although a role of c-Src in EGF activation of ERK5 has not yet been demonstrated, these studies provide the background for a potential role of ERK5 in the c-Src-mediated activation of HER1. One substrate of ERK5 is the early response gene *c-myc* [59]. *C-myc* encodes a nuclear phosphoprotein, which, in combination with Max, activates gene transcription. C-Myc expression correlates with the proliferative state [60], and has been shown to rescue platelet-derived growth factor signaling that is blocked by kinase-inactive c-Src [61]. Together these findings link c-Src and EGF with PI-3K, ERK5, and c-Myc, and are suggestive of a potential role for one or more of these molecules to function as downstream effectors of phosphorylated Tyr 845.

Conclusion

Substantial evidence is accumulating to indicate functional synergism between the nonreceptor tyrosine kinase c-Src and members of the EGF receptor family in promoting breast cancer progression. Members of both families are overexpressed in approximately 70% or more of human breast cancers, and the human EGF receptor (HER1) and HER2/*neu* portend a poor prognosis for the disease.

It has been demonstrated that c-Src, which is nontransforming when overexpressed alone, can potentiate the

tumorigenic capacity of overexpressed HER1. Recently, one mechanism by which c-Src synergizes with the HER1 has been uncovered. This mechanism involves the EGF-dependent association of c-Src with HER1 and phosphorylation of the receptor by c-Src on residues Tyr 845 and Tyr 1101. The functional consequences of Tyr 1101 phosphorylation are unknown, but phosphorylation of Tyr 845 is required for EGF-induced DNA synthesis and activation of members of the STAT family of transcription factors, particularly STAT5b, but not activation of Shc or MAPK. Whether the STATs are the predominant mediators of Tyr 845-dependent mitogenesis or whether there are other mitogenic signaling pathways that emanate from phosphorylated Tyr 845 remains to be determined. Surprisingly, Tyr 845 phosphorylation has also been found to be an intermediate in mitogenic signaling from a variety of GPCR, as well as from certain cytokine receptors and the estrogen receptor. Thus, HER1, and specifically phosphorylation of Tyr 845 by c-Src, appears to play an important, perhaps widespread role in mediating cell responses to an array of external signals.

c-Src also complexes with another member of the EGF receptor family, namely HER-2 or *erbB-2/neu*. This association is independent of extracellular ligand and appears to contribute more to cell survival and anchorage-independent growth of breast cancer cells than to anchorage-dependent growth or migration. The mechanism of c-Src interaction with HER2 and how this interaction may transmit survival or anchorage-independent growth signals is not known, but it is speculated to be different than the interaction between c-Src and HER1.

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c-Src, Receptor Tyrosine Kinases, and Human Cancer

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I. INTRODUCTION

Since the discovery that tyrosine kinases are among the transforming proteins encoded by oncogenic animal retroviruses, it has been speculated that this family of enzymes may contribute to the development of human malignancies. However, evidence supporting that hypothesis has been slow to evolve, largely because early emphasis was placed on examining human tumors for genetic alterations in protooncogenes encoding these enzymes. Such alterations have proved rare or nonexistent. Instead, investigations have fo-

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cused on determining levels of expression and posttranslational mechanisms of regulation of these proteins, particularly as they relate to signaling pathways that modulate growth, adhesion, invasion, and motility. Two classes of tyrosine kinases have emerged as potentially important players in promoting the evolution of human tumors: receptor kinases (RTKs) and nonreceptor tyrosine kinases of the c-Src family. Elevated levels of both these classes of tyrosine kinases can be found in a large number of tumors in a strikingly similar pattern of aberrant cooverexpression, suggesting that the two families may cooperate with one another during oncogenesis. Indeed, in model tissue culture systems, overexpression of receptor alone can result in malignant transformation when a continuous source of ligand is provided. However, overexpression of c-Src alone is non- or weakly oncogenic. These results indicate that c-Src, if it plays a role in tumorigenesis, most likely mediates its effects through RTKs. Demonstrations that c-Src physically associates with a number of RTKs in a ligand-dependent fashion provided some of the first evidence for functional cooperativity between these families of proteins. Subsequent studies showed that in complex, the two kinases reciprocally affect one another's behavior, such that c-Src can be regarded both as a regulator of RTKs and as a cotransducer of signals emanating from them. c-Src is capable of physically associating with the receptors for platelet-derived growth factor (PDGF), prolactin, epidermal growth factor (EGF), colony-stimulating factor-1 (CSF-1), fibroblast growth factor (FGF), and hepatocyte growth factor/scatter factor (HGF/SF), as well as with the HER2/*neu* and Sky tyrosine kinases (this review and Toshima *et al.*, 1995; Berlanga *et al.*, 1995), all of which are postulated to play a role in the genesis and/or progression of various human cancers. Although c-Src and its family members are also known to participate in signaling events elicited by heterotrimeric G protein-coupled receptors (Malarkey *et al.*, 1995) and neuronal ion channels (Ely *et al.*, 1994; Holmes *et al.*, 1996; Yu *et al.*, 1997; van Hoek *et al.*, 1997), this review focuses on the interactions of c-Src and Src family members with RTKs because of the growing documentation of the interactions between these proteins in human malignancies. First, a summary is presented, naming the RTKs that are most frequently implicated etiologically in human cancers and that have been shown to interact with c-Src. This summary includes a short review of the physical characteristics of the receptors, their molecular mechanisms of signaling, and their putative roles in specific cancers. Second, evidence is discussed for the involvement of c-Src and Src family members in human tumor development, and third, a synopsis is outlined showing the molecular mechanisms by which c-Src and its family members have been found to interact with receptors and other targets. Finally, we will speculate on the prospects for developing novel therapies based on these interactions.

II. RECEPTOR TYROSINE KINASES AND HUMAN CANCERS

Figure 1 depicts the structural features of several classes of RTKs that interact with c-Src. All consist of an extracellular ligand-binding domain that bears motifs characteristic of the type of receptor (e.g., repeated immunoglobulin-like motifs for the PDGF and FGF receptors or cysteine-rich motifs in the EGF family of receptors), a transmembrane segment, a tyrosine

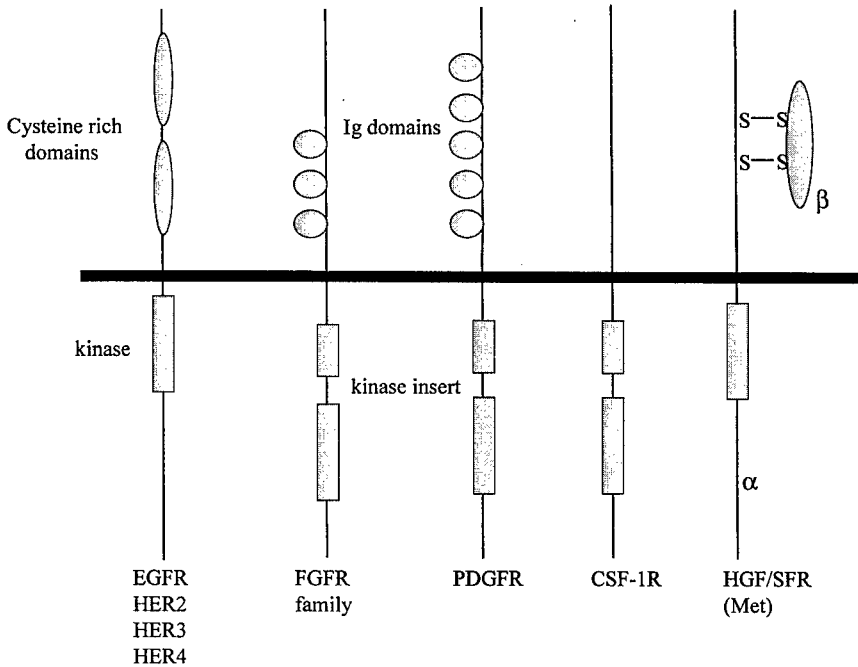


Fig. 1 Structures of receptor tyrosine kinase families known to associate with c-Src. All receptors are transmembrane glycoproteins that function as receptors for polypeptide growth factors. Structurally, these molecules are composed of large, extracellular domains that exhibit characteristic ligand-binding motifs, as well as transmembrane, juxtamembrane, catalytic, and C-terminal domains. Ligand binding induces dimerization, enzymatic activation, and autophosphorylation on specific tyrosine residues in the C-terminal domains. These phosphorylated tyrosine residues serve as docking sites for signaling molecules that transmit biological signals from the extracellular milieu to the nucleus. In the platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) receptor families, the kinase domain is interrupted by an insert that contains additional docking sites. EGFR, Epidermal growth factor receptor; CSF-1R, colony-stimulating factor-1 receptor; HGF/SFR, hepatocyte growth factor/scatter factor receptor; HER, human epidermal growth factor receptor.

kinase catalytic domain, and a carboxy-terminal region that contains sites of autophosphorylation. Binding of ligand causes dimerization of the receptor, activation of tyrosine kinase activity, and (trans) autophosphorylation of specific C-terminal tyrosine (Tyr) residues (reviewed in Heldin, 1996; Weiss *et al.*, 1997), which in turn serve as docking sites for a variety of signaling molecules that contain SH2 domains (Pawson and Schlessinger, 1993), including phospholipase C γ (PLC γ), phosphatidylinositol-3 kinase (PI-3) kinase), GTPase-activating protein of Ras (RasGAP), phosphotyrosine phosphatases (PTPases), Janus kinases/signal transducers and activators of Transcription (JAK/STATS), adapter proteins (including Shc, Grb, Nck), and members of the c-Src family of tyrosine kinases (reviewed in Erpel and Courtneidge, 1995; Heldin, 1996). Signals are subsequently transmitted to the nucleus via several pathways, including the JAK/STAT and the Grb2/SOS/Ras/Raf/MEK/MAP kinase cascades (reviewed in Bonfini *et al.*, 1996; Denhardt, 1996). Members of the STAT and MAP kinase families translocate from the cytoplasm to the nucleus and induce changes in gene expression, which bring about a variety of functional outcomes, such as mitogenesis, morphogenesis, and motility. The contribution of c-Src to downstream signaling from these RTKs has been the subject of growing interest, with emphasis on how c-Src may contribute to transformation and maintenance of the cancerous phenotype that is dependent on and induced by the receptors.

In this treatise, a total of five RTK families and their putative roles in development of malignancy will be considered. The first four, receptors for HGF/SF, CSF-1, FGF, and PDGF, are implicated as etiological agents in a wide variety of human cancers, and their ability to influence processes such as cytoskeletal changes, cell motility, and angiogenesis are thought to contribute to the metastatic potential of tumors. The fifth group, members of the EGF receptor family (HER1–4), will be discussed in the context of breast cancer, along with the estrogen receptor. This steroid hormone receptor plays a pivotal role in the etiology of breast cancer and growing evidence indicates its ability to reciprocally interact with c-Src and members of the HER family of RTKs.

A. Hepatocyte Growth Factor/Scatter Factor Receptor

The Met tyrosine kinase is the receptor for hepatocyte growth factor/scatter factor (Bottaro *et al.*, 1991; Naldini *et al.*, 1991). This receptor was first identified as the product of the human oncogene, *tpo-met*, which was isolated from a chemically treated human cell line by the NIH3T3 gene transfer method (Cooper *et al.*, 1984; Park *et al.*, 1987). The normal cellular receptor is composed of two subunits, a 145-kDa β chain, which spans the cell

membrane and possesses ligand-binding and tyrosine kinase activity, and a 50-kDa α chain, which resides extracellularly and is covalently bound to the β subunit through disulfide linkages (Gonzatti-Haces *et al.*, 1988). Related family members include the Sea and Ron RTKs (Ronsin *et al.*, 1993; Huff *et al.*, 1993). Each member of the Met family possesses two tandemly arranged, degenerate YVH/NV motifs in the C-terminal tail of the receptor, which are capable of binding the SH2 domains of the signaling molecules PI-3 kinase, PTPase 2, PLC γ , c-Src, and Grb2/Sos (Ponzetto *et al.*, 1994). Mutations in these motifs (H1351N) result in increased transforming ability but decreased metastasis (Giordano *et al.*, 1997), a phenomenon that is linked to the creation of an additional Grb2 binding site and hyperactivation of the Ras pathway.

HGF/SF is produced by cells of mesodermal origin and acts on epithelial and endothelial cells, eliciting numerous biological responses, including cell motility, growth, morphogenesis, differentiation, and angiogenesis (Kan *et al.*, 1991; Rubin *et al.*, 1991; Halaban *et al.*, 1992). Which response is elicited in part depends on the cell type, developmental stage, and tissue context (Weidner *et al.*, 1993; Kanda *et al.*, 1993; Zhu *et al.*, 1994; Rosen and Goldberg, 1995; Grano *et al.*, 1996). For example, Met signals through STAT3 to induce the formation of branched tubule structures in Madin-Darby canine kidney (MDCK) cells, a hallmark of angiogenesis (Boccaccio *et al.*, 1998). HGF binding to primary human osteoclasts and osteoblasts triggers receptor kinase activity and autophosphorylation in both cell types. However, in osteoclasts, HGF binding is accompanied by increased levels of intracellular calcium, activation of c-Src, changes in cell shape, stimulation of chemotaxis, and DNA replication, whereas osteoblasts respond simply by undergoing DNA synthesis (Grano *et al.*, 1996). Furthermore, osteoclasts also express HGF, but osteoblasts do not. This finding suggests that an autocrine loop may be responsible for signaling in osteoclasts, whereas a paracrine mechanism is functional in osteoblasts.

HGF/SF and the Met receptor have been implicated in several types of human cancer. Met is overexpressed in gastric, ileal, colorectal, and thyroid papillary carcinomas, as well as in osteogenic sarcoma (Di Renzo *et al.*, 1991, 1992; Rosen *et al.*, 1994; Grano *et al.*, 1996). The level of Met expression, as measured by intensity of Met immunofluorescence, has also been shown to correlate with grade of malignancy in primary human brain tumors (Koochekpour *et al.*, 1997). In the case of ovarian carcinoma, Met levels can be regulated by the cytokines interleukin 1 α (IL-1 α), IL-6, and tumor necrosis factor α (TNF α), thereby providing a physiological mechanism by which overexpression of Met can be achieved (Moghul *et al.*, 1994). Approximately 14% of patients with papillary renal carcinoma have germ-line alterations in the Met receptor (Schmidt *et al.*, 1997). Receptors bearing these mutations have been shown in NIH3T3 cells to result in increased tyrosine kinase activity of the receptors and Met-mediated focus formation

and tumors in nude mice, thus providing direct evidence for the ability of mutationally altered Met to function as an oncogene (Jeffers *et al.*, 1997).

The ability of HGF/SF to "scatter" cells and to increase their motility is strongly suggestive of a role for this ligand in tumor cell invasion. Indeed, several lines of evidence link HGF/SF to stimulation of the urokinase plasminogen activator (UPA) system, a cascade of proteases thought to promote release, extravasation, and migration of tumor cells. That the UPA cascade is critical for cell migration is supported by the findings that UPA $-/-$ mice are unable to recruit migrating cells in response to inflammation (Gyetko *et al.*, 1996), and do not support the growth and metastasis of experimental melanomas (Min *et al.*, 1996). Shapiro *et al.* (1996) also showed that blocking interaction of UPA with its receptor results in decreased angiogenesis and tumor spread. The link between UPA and HGF was made when Jeffers *et al.* (1996b) reported that stimulation of the urokinase proteolytic system occurred concomitantly with HGF/SF-induced invasion and metastasis of human tumor cells. Rosen and Goldberg (1995) also demonstrated that HGF/SF is capable of stimulating angiogenesis in a rat cornea neovascularization assay. Together, these studies provide compelling evidence that HGF/SF are capable of promoting tumor progression by enhancing invasion and angiogenesis.

Further evidence for a role for HGF/S/Met receptor in tumor invasiveness and angiogenesis comes from the findings that high titers of HGF/SF in invasive breast cancers are factors for relapse and death (Yamashita *et al.*, 1994), that HGF/SF treatment of glioma cell lines stimulates proliferation and invasion (Koochekpour *et al.*, 1997), and that invasive bladder carcinomas possess higher HGF/SF titers than do noninvasive cancers (Joseph *et al.*, 1995). In addition, the Met receptor is overexpressed in several types of tumor stroma, including bladder wall, vascular smooth muscle, and vascular endothelial cells (Rosen and Goldberg, 1995), suggesting a paracrine signaling mechanism between tumor cells and the underlying stroma. Thus, HGF/SF and Met interactions may promote metastasis by enhancing proliferation via autocrine or paracrine routes, stimulating the expression of plasminogen activators, and triggering angiogenesis. (Rong *et al.*, 1992; Kanda *et al.*, 1993; Bellusci *et al.*, 1994; Jeffers *et al.*, 1996a,b).

B. Colony-Stimulating Factor-1 Receptor

c-Fms, the cellular homolog of the viral oncogene v-Fms (Sherr *et al.*, 1985), is the receptor for colony-stimulating factor-1, which stimulates the proliferation and differentiation of macrophages, osteoclasts, and placental trophoblasts (Sherr, 1990; Roth and Stanley, 1992; Insogna *et al.*, 1997). That CSF-1 is critical for the development of mononuclear phagocytes was

shown by studies in mice that fail to express functional CSF-1: these mice exhibit an osteopetrotic phenotype and lack osteoclasts and macrophages (Wiktor-Jedrzejczak *et al.*, 1990, 1991). The CSF-1 receptor is expressed in placenta (Pollard *et al.*, 1987; Regensstreif and Rossant, 1989; Hume *et al.*, 1997), osteolasts (Insogna *et al.*, 1997), and cells of monocyte lineage (Woolford *et al.*, 1985), whereas the ligand, CSF-1, is produced by fibroblasts, myoblasts, osteoblasts, bone marrow stromal cells, and endothelial cells (Sherr, 1990; Roth and Stanley, 1992). Such independent distribution of ligand and receptor underlies the importance of cell-cell interactions in regulating receptor function.

c-Fms bears sequence and structural similarity to the *steel* receptor, c-Kit, and to the receptors for FGF, PDGF, and Flt3/FLK2 (Hanks *et al.*, 1988; Rosnet and Birnbaum, 1993). The unique feature of this group is that each member possesses an "insert" region within its kinase domain. The downstream targets of c-Fms include PI-3 kinase, STAT 1, and PLC γ , all of which bind to phosphorylated tyrosine residues within the kinase insert portion of the molecule (Varticovski *et al.*, 1989; Shurtleff *et al.*, 1990; Reedijk *et al.*, 1990; Novak *et al.*, 1996; Bourette *et al.*, 1997). Bourette *et al.* (1997) have shown that sequential activation of the PI-3-kinase-dependent and PLC γ -dependent signaling pathways is required to initiate the differentiation process of myeloid cells. c-Src has also been shown to associate with c-Fms and to be activated on binding of CSF-1 to the receptor. Complex formation between c-Src and c-Fms is thought to occur via the SH2 domain of c-Src and a juxtamembrane phosphotyrosyl residue on the receptor (Courtneidge *et al.*, 1993; Alonso *et al.*, 1995). In osteoclasts, phosphorylation of c-Src in response to CSF-1 stimulation occurs concomitantly with rearrangements of the actin cytoskeleton and spreading of the cells, suggesting that c-Src may be involved in regulating these processes (Insogna *et al.*, 1997).

Overexpression of c-Fms in NIH3T3 or Rat2 fibroblasts or in various tumor cells results in transformation, growth in soft agar, and tumor formation in nude mice (Rettenmeier *et al.*, 1987; Taylor *et al.*, 1989; van der Geer and Hunter, 1989; Favot *et al.*, 1995). These findings demonstrate the oncogenic potential of overexpressed c-Fms. As described above, c-Fms and CSF-1 are normally not expressed in the same cell type. However, coexpression is seen in tumors of the pancreas, endometrium, stomach, lung, and breast, and in acute myeloid leukemia, hairy cell leukemia, and Hodgkin's lymphoma (Rambaldi *et al.*, 1988; Kacinski *et al.*, 1990; Paietta *et al.*, 1990; Baiocchi *et al.*, 1991; Kauma *et al.*, 1991; Bruckner *et al.*, 1992; Filderman *et al.*, 1992; Storga *et al.*, 1992; Tang *et al.*, 1992; Leiserowitz *et al.*, 1993; Till *et al.*, 1993; Burthem *et al.*, 1994; Berchuck and Boyd, 1995). Coexpression correlates with poor patient prognosis, most likely due to the establishment of an autocrine loop (Kacinski *et al.*, 1990; Tang *et al.*, 1992). Evidence suggests that such an autocrine loop contributes not only to tumor

cell proliferation but also to invasiveness (Bruckner *et al.*, 1992; Filderman *et al.*, 1992; Burthem *et al.*, 1994). In this regard, coexpression of c-Fms and its ligand in endometrial cancers correlates with a more advanced stage and with increased myometrial invasion (Leiserowitz *et al.*, 1993). Moreover, CSF-1 stimulation results in the expression of UPA in lung tumors, Lewis lung carcinoma cells, and in NIH3T3 cells transfected with c-Fms (Filderman *et al.*, 1992; Favot *et al.*, 1995; Stacey *et al.*, 1995). Together, these findings suggest that, like HGF/SF/MetR, deregulation of c-Fms/CSF-1 interactions has the potential of contributing to the metastatic process in a variety of human cancers.

C. Fibroblast Growth Factor Receptors

The FGF receptors comprise a large family that is encoded by four separate genes, each of which can be alternatively spliced. Each receptor is also capable of binding several different ligands, resulting in a complex array of possible receptor/ligand pairs (Johnson and Williams, 1993). All receptors for FGF possess extracellular ligand-binding domains, which contain immunoglobulin-like repeats, and bipartite, intracellular tyrosine kinase domains (Lappi, 1995). Which signaling molecules are recruited varies with cell type and receptor/ligand pair. For example, in NIH3T3 cells (Zhan *et al.*, 1994) FGFR 1 and c-Src physically associate following ligand binding, and activation of the receptor triggers the c-Src-dependent phosphorylation of the actin-binding protein, cortactin. Because cortactin is localized to cortical actin, particularly at the leading edge of a migrating cell (Wu *et al.*, 1991; Maa *et al.*, 1992; Wu and Parsons, 1993), its phosphorylation is speculated to influence cell motility and invasiveness. In other studies, ligand stimulation of FGFR 1 and FGFR 3 on C6 rat myoblasts results in activation of the p21Ras and MAPK pathway (Klint *et al.*, 1995; Kanai *et al.*, 1997). In these same cells, activation of the FGFR 3 receptor alone causes an increase in phosphorylation of PLC γ but a decrease in c-Src phosphorylation (Kanai *et al.*, 1997).

FGF receptors are ubiquitously expressed during embryogenesis, but their presence is restricted after birth (Wanaka *et al.*, 1991; Peters *et al.*, 1992, 1993; Pastone *et al.*, 1993). As a family, FGFs have mitogenic, nonproliferative, and antiproliferative effects. Which response is elicited is determined by the ligand, the type of cell exposed to the ligand, and the particular isoform of the receptor expressed on that cell (Schweigerer *et al.*, 1987; Sporn and Roberts, 1988). For example, FGF 2 promotes survival of cultured neurons (Walicke, 1988), whereas FGF 1 and FGF 2 stimulate growth of fibroblasts, oligodendrocytes, astrocytes, smooth muscle cells, endothelial

cells, and retinal epithelial cells (Burgess and Maciag, 1989). FGFs can also act as chemotactic factors for fibroblasts and glial cells (Senior *et al.*, 1986). Basic FGF (bFGF, or FGF 2) induces neurite outgrowth in embryonic chick ciliary ganglion cells (Schubert *et al.*, 1987) and can mediate cellular migration in experimental systems (Sato and Rifkin, 1988). Treatment of cultured vascular endothelial cells with FGF 2 induces the formation of blood capillary-like tubules, a finding that suggests FGFs may play a role in angiogenesis (Montesano *et al.*, 1986; Slavin, 1995). In this regard, a large literature is beginning to accumulate in support of a role for FGFs in angiogenesis, because they have been demonstrated to stimulate endothelial cell division, migration, release of proteolytic enzymes, and capillary formation (Slavin, 1995).

In addition to these functions in normal cells, FGFR family members are implicated in the progression of a variety of human cancers. FGFs are thought to act as autocrine growth factors for melanomas, gliomas, and meningiomas (Lappi, 1995), and their levels are elevated in many different tumor types (Nguyen *et al.*, 1994). FGF receptors are also overexpressed in human tumors. For example, 10% of human breast tumors exhibit amplifications of chromosomal regions encoding FGF receptors (Adnane *et al.*, 1991), and FGFR 4 mRNA levels are frequently elevated in breast cancer cells as compared to normal tissue (Lehtola *et al.*, 1993; Ron *et al.*, 1993; Penault-Llorca *et al.*, 1995). Some evidence also suggests that differential expression of FGFR isoforms can influence the propensity of a cell to undergo malignant transformation. In normal fetal and mature brain, FGFR 1, which possesses three immunoglobulin-like extracellular repeats, is expressed. However, in astrocytic tumors, an increase in the expression of an FGFR with two immunoglobulin-like domains is observed. This form has increased affinity for acidic and basic FGF (Shing *et al.*, 1993). Changes in FGFR expression also occur during the conversion of normal or hyperplastic prostatic epithelium to malignant tumor tissue, where the increased expression of an alternatively spliced form of FGFR 2, which has a higher affinity for bFGF, appears to create an autocrine stimulatory loop (Wang *et al.*, 1995).

FGFs, along with other factors, are often secreted by tumors, and their increased extracellular abundance is linked to enhanced invasiveness (Klagsbrun *et al.*, 1976; Libermann *et al.*, 1987; Wadzinski *et al.*, 1987; Folkman *et al.*, 1988). In breast tumor cells, FGFR 4 activation results in membrane ruffling, a morphological change that is associated with metastasis (Johnston *et al.*, 1995). In *in vitro* invasion assays, FGF 2 induces the migration of bovine capillary endothelial cells through placental tissue in a dose-dependent manner (Mignatti *et al.*, 1989), and bFGF stimulates production of metalloproteinases in human bladder cancer cell lines, an event associated with increased invasiveness of the cells (Miyake *et al.*, 1997). Moreover,

bFGF-dependent, sustained activation of MAPK correlates with the scattering of neuroepithelioma cells (van Puijenbroek *et al.*, 1997). Together, these studies suggest that FGFs and FGFRs play important roles in human cancer progression by promoting the metastatic process.

D. Platelet-Derived Growth Factor Receptor

The PDGFR has two isoforms, α and β , which differ in their preferences for binding homo- or heterodimers of the A and B forms of the PDGF ligand (Yarden *et al.*, 1986; Claesson-Welsh *et al.*, 1989; Claesson-Welsh and Heldin, 1989; Heldin and Westermark, 1990; Ross *et al.*, 1990; Matsui *et al.*, 1993). Both receptor isoforms consist of an extracellular domain that contains immunoglobulin-like motifs, transmembrane and juxtamembrane regions, a catalytic domain with an insert, and a C-terminal tail (Heldin and Westermark, 1990; Ross *et al.*, 1990). Signaling molecules, which include PI-3 kinase (Kazlauskas and Cooper, 1989; Auger *et al.*, 1989; Coughlin *et al.*, 1989), PLC γ (Kumjian *et al.*, 1989; Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989; Morrison *et al.*, 1990), RasGAP (Molloy *et al.*, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990), and the Src family members c-Src, Fyn, and c-Yes (Kypta *et al.*, 1990) (Twamley *et al.*, 1992), bind phosphorylated tyrosine residues in the C-terminal tail, the kinase insert, and the juxtamembrane region via their SH2 domains.

Interestingly, the same downstream effectors in different cell types can elicit different cellular responses. For example, in human hepatoma cell lines, PLC γ and PI-3 kinase can independently transmit mitogenic signals (Valius and Kazlauskas, 1993), whereas in CHO cells a precise balance exists between migration-promoting signaling via PLC γ and PI-3 kinase and migration-inhibitory signaling via RasGAP (Kundra *et al.*, 1994). Phosphorylation of Tyr-988 in the carboxy terminus of the α receptor is associated with induction of chemotaxis, whereas phosphorylation of Tyr-768 and Tyr-1018 negatively regulates this process (Yokote *et al.*, 1996). These results suggest that the different phosphorylation sites serve as binding sites for unique signaling molecules that influence cellular behavior in different ways. This hypothesis is further supported by studies in smooth muscle cells showing that PDGF-induced activation of PLC γ is associated with actin disassembly and chemotaxis, whereas an independent signaling pathway, probably involving small GTPases such as Rho, appears to mediate the proliferative effect of PDGF in this system (Bornfeldt *et al.*, 1995).

PDGF receptors and their ligands regulate a wide spectrum of normal cellular processes in cells of mesenchymal and endothelial origin. These processes include differentiation, proliferation, survival, and migration. For example, the receptor for PDGF α is necessary for the development of neur-

al crest cells (Soriano, 1997) and alveolar branching in the lung (Souza *et al.*, 1995); the PDGF β receptor is required for proper development of the cardiovascular and renal systems (reviewed in Betsholtz, 1995). The PDGF β receptor is also found in mesenchymal tissue of the developing trachea and intestine and in the endothelium of blood vessels, where it is thought to play a role in regulating mesenchymal-epithelial interactions (Shinbrot *et al.*, 1994). In addition, the PDGF α receptor is required for the maximal chemotactic effect of PDGF on lung fibroblasts (Osornio-Vargas *et al.*, 1996).

Numerous studies suggest that various PDGF and PDGFR isoforms are also involved in the genesis or maintenance of human cancers. The PDGFR is overexpressed in human pancreatic cancer (Ebert *et al.*, 1995), primary and metastatic melanomas (Barnhill *et al.*, 1996), and in mesothelioma cell lines (Versnel *et al.*, 1994; Langerak *et al.*, 1996). PDGFR expression is also seen in many neural crest-derived human tumors, including neuroblastoma and Ewing's sarcoma (Matsui *et al.*, 1993), in basal cell carcinoma (Ponten *et al.*, 1994), and in tumors of the lung and pituitary (Leon *et al.*, 1994; Vignaud *et al.*, 1994). PDGF and its receptors are not normally expressed in epithelial cells, but their aberrant expression in tumors of this origin suggest that they could be involved in the oncogenic process.

The situation is made more complex by the fact that some tumors express one or both forms of the ligand and no receptor(s) or vice versa, suggesting that both autocrine and paracrine signaling loops are involved in PDGF-mediated growth of tumors. For example, autocrine signaling loops have been shown to contribute to the growth of human esophageal carcinomas (Juang *et al.*, 1996), mesotheliomas (Langerak *et al.*, 1996), malignant melanomas (Barnhill *et al.*, 1996), gliomas, and glioblastomas (Potapova *et al.*, 1996). However, results from Coltrera *et al.* (1995) show that PDGF may also function in a paracrine fashion in some human breast tumors. Their studies revealed that PDGF β is expressed in breast epithelium and tumor tissues, and the receptor is present in stromal fibroblasts. A similar situation appears to exist in ovarian cancer (Versnel *et al.*, 1994), in lung tumors (Vignaud *et al.*, 1994), and in basal cell carcinomas (Ponten *et al.*, 1994). The ability of PDGF to induce chemotaxis may also play a role in tumor cell metastasis. For example, expression of the receptor for PDGF α in Lewis lung carcinoma cells increases their metastatic potential, whereas expression of the receptor truncated at the kinase domain reverses this effect (Fitzer-Attas *et al.*, 1997). Potapova *et al.* (1996) demonstrated that in human glioblastoma cells, which express both the PDGF β receptor and its ligand, further expression of PDGF β results in tumor formation in nude mice and increased metastasis. These examples support the idea that in addition to mediating normal cell migration, aberrant expression or activation of PDGF receptors in tumor cells can contribute to their proliferative and invasive properties.

E. Epidermal Growth Factor Receptor

The human epidermal growth factor receptor, HER1, belongs to a family of human RTKs that includes HER2/*neu*, HER3, and HER4 (Ullrich and Schlessinger, 1990). All members of this family are transmembrane tyrosine kinases that possess an extracellular domain with two cysteine-rich repeats, an intact catalytic domain, and a C-terminal tail that binds SH2-containing signaling effectors on activation of the receptor. Ligands for HER1 include epidermal growth factor (EGF), transforming growth factor- α (TGF- α), betacellulin (Riese *et al.*, 1996), and epiregulin (Komurasaki *et al.*, 1997). No specific ligand for HER2 has yet been defined, but HER3 and HER4 can be activated by a family of alternatively spliced ligands, called heregulins (HRG) (reviewed in Hynes and Stern, 1994). Each member of the HER family is capable of heterodimerizing with other members of the family, thereby providing a means by which HER2, although it lacks a ligand, can signal. Such dimerization appears to occur in a hierarchical order, wherein the HER2/3 interaction is the most preferred and the HER1/4 interaction the least preferred (Pinkas-Kramarski *et al.*, 1996). Studies in 32D hematopoietic cells, which do not express any HER family members, show that heterodimers have more potent mitogenic activity than do homodimers and that HER3 heterodimers are the most transforming. However, when HER1 is present, signaling through this receptor dominates over other members of the family (Pinkas-Kramarski *et al.*, 1996).

The focus of our discussion will be on the roles of HER1 and HER2 in human cancer, because large bodies of literature exist for each. HER3 and HER4 are more recent additions to the family, and characterization of them with respect to their possible involvement in human cancers is just beginning. However, it is important to note that overexpression of HER3 has been detected in some breast cancers (Lemoine *et al.*, 1992) and in papillary thyroid carcinomas (Faksvag *et al.*, 1996). Thus, the possibility exists that homo- or heterodimerization of HER3 or HER4 with HER1 or HER2 mediates tumorigenic signaling in a manner similar to that of HER1 and HER2.

A major role for HER1 is its involvement in normal human development. It affects many stages, from postfertilization to sexual maturation. For example, HER1 and its ligand, TGF- α , control proliferation of blastocoel cells as well as embryo/uterine signaling and implantation (Rappoll *et al.*, 1988; Dardik and Schultz, 1991; Arnholdt *et al.*, 1991; Zhang *et al.*, 1992). HER1 is also necessary for development of embryonic lung, skin, palate (Lee and Han, 1990), and hair follicles (Hansen *et al.*, 1997). During puberty, HER1 and the estrogen receptor together regulate the differentiation of normal breast epithelium and uterine and vaginal growth (Nelson *et al.*, 1991; Ignar-Trowbridge *et al.*, 1992). Loss of control of these interactions is thought to play a role in the genesis of human tumors, and the diversity of tissues

that are regulated developmentally by HER1 is reflected in the spectrum of tissues and cell types in which HER1 is thought to play an oncogenic role.

That HER1 can function as an oncoprotein was demonstrated by the ability of NIH3T3 cells, engineered to overexpress HER1 and held in the continual presence of EGF, to become transformed and develop tumors in nude mice (Velu *et al.*, 1987; DiFiore *et al.*, 1987a; Di Marco *et al.*, 1989). HER1 effector substrates include the adaptor proteins Shc (Pelicci *et al.*, 1992; Ruff-Jamison *et al.*, 1993) and Grb2, which feed into the well-defined Ras/MAPK signaling pathway (Li *et al.*, 1993; Egan *et al.*, 1993; Rozakis-Adcock *et al.*, 1993), as well as PLC γ (Rhee, 1991), c-Cbl (Levkowitz *et al.*, 1996), eps 8, and eps 15 (Fazioli *et al.*, 1992, 1993a,b). Reports from several laboratories show that on activation, HER1 physically associates with the c-Src nonreceptor tyrosine kinase in both normal fibroblasts and in a variety of tumor cell lines (Luttrell *et al.*, 1994; Maa *et al.*, 1995; Sato *et al.*, 1995; Stover *et al.*, 1995; Biscardi *et al.*, 1998a). Complex formation with c-Src occurs concomitantly with enhanced phosphorylation of receptor substrates, suggesting that c-Src may act to increase the receptor's tyrosine kinase activity, thus enhancing the potential for cellular transformation and tumorigenesis (Maa *et al.*, 1995; Tice *et al.*, 1998; Biscardi *et al.*, 1998a,b). This hypothesis was tested directly using a panel of C3H10T1/2 murine fibroblasts that were engineered to overexpress HER1 and c-Src, either alone or in combination. Cells overexpressing both HER1 and c-Src were found to produce synergistically larger and more numerous tumors in nude mice and colonies in soft agar than those produced by cells overexpressing either HER1 or c-Src alone (Maa *et al.*, 1995). These findings represent the first causal evidence for cooperativity between c-Src and HER1 in tumorigenesis.

What is the evidence for involvement of HER1 in the genesis of human tumors? Aberrant expression, overexpression, or truncation of HER1 has been demonstrated to occur in a variety of human cancers, including benign skin hyperplasia, glioblastoma, and cancers of the breast, prostate, ovary, liver, bladder, esophagus, larynx, stomach, colon, and lung (Harris *et al.*, 1992; Khazaie *et al.*, 1993; Scambia *et al.*, 1995). In patients with ovarian cancer, overexpression of HER1 correlates with a decreased response to chemotherapy and decreased survival (Scambia *et al.*, 1995; Fischer-Colbrie *et al.*, 1997), suggesting that HER1 plays a proactive role in ovarian tumor progression.

HER1 overexpression also appears to play a role in the etiology of glioblastomas. Forty percent of glioblastomas exhibit amplification of the HER1 gene (Khazaie *et al.*, 1993), but in these tumors, overexpression is not the only abnormality regarding HER1. An alternatively spliced form of the receptor, termed EGFRvIII, is also frequently observed (Libermann *et al.*, 1985; Yamazaki *et al.*, 1988; Tuzi *et al.*, 1991; Chaffanet *et al.*, 1992). This form of the receptor lacks nucleotides 275–1075, which encode a large

portion of the extracellular domain (Humphrey *et al.*, 1990; Ekstrand *et al.*, 1992; Wong *et al.*, 1992, and displays constitutive activity, perhaps due to its inability to be controlled by ligand (Ekstrand *et al.*, 1994). Existing evidence suggests that EGFRvIII signals differently than wild-type receptor, preferring the PI-3 kinase pathway (Moscattello *et al.*, 1998) to the Ras/MAPK pathway (Montgomery *et al.*, 1995; Moscattello *et al.*, 1998). In addition to glial tumors, one study showed that EGFRvIII is present in 16% of non-small-cell lung carcinomas (Garcia *et al.*, 1993) as well as in 86% of medulloblastomas, 78% of breast cancers, and 73% of the ovarian cancers examined (Moscattello *et al.*, 1995). In contrast, EGFRvIII has not yet been detected in normal tissue, a finding that provides compelling evidence for an oncogenic role for this form of the receptor.

A link between HER1 and breast cancer has also emerged in recent years. Amplification or overexpression of the genes encoding one or more of the HER family members is estimated to occur in approximately 67% of human breast cancers (Harris *et al.*, 1992), with overexpression of HER1 detected in approximately 30% of patients (Battaglia *et al.*, 1988; Delarue *et al.*, 1988; Bolla *et al.*, 1990; Koenders *et al.*, 1991; Toi *et al.*, 1991; Harris *et al.*, 1992). Elevated levels of HER1 are also associated with loss of estrogen-dependent growth (Klijn *et al.*, 1993), suggesting a role for HER1 in the later stages of tumor progression.

In addition to transformation and proliferation, studies from several laboratories suggest that HER1 also enhances the invasive potential of tumor cells. Overexpression of HER1 has been shown to result in an increased ability of rat mammary carcinoma cells to migrate through matrigel (Lichtner *et al.*, 1995; Kaufmann *et al.*, 1996), and higher levels of HER1 are found in tumor tissue at metastatic sites as compared to primary sites (Sainsbury *et al.*, 1987; Toi *et al.*, 1991). Both these findings are supportive of a role for HER1 in metastasis.

F. HER2/*neu*

Like HER1, activated HER2 possesses an intracellular tyrosine kinase domain as well as C-terminal phosphotyrosines that are capable of binding downstream substrates, such as PLC γ , PI-3 kinase, Grb7, p120 RasGAP, p190, RhoGAP, c-Src, Shc, PTP1D, PTP1B, eps-8, and Tob, an antiproliferative protein (Hynes and Stern, 1994; Matsuda *et al.*, 1996; Liu and Chernoff, 1997). Because both HER1 and HER2 appear to activate similar downstream signaling pathways in experimental cell systems, it is unclear how specificity of signaling is achieved. The most likely explanation is that activation of a particular signaling pathway is dependent on cell type and on the

subset of HER family members and effector molecules available at any given time. However, a few examples of specific substrates have been reported, such as the c-Cbl adaptor protein for HER1 (Levkowitz *et al.*, 1996) and paxillin and a protein of 23 kDa (p23) for HER2 (Romano *et al.*, 1994).

HER2 is expressed in all tissues except the hematopoietic system (De Potter *et al.*, 1990; Press *et al.*, 1990). Studies using mice that are deficient in HER2, HER4, or the HER3/4 ligand, HRG, demonstrate that signaling through HER2 heterodimers is necessary for proper cardiac and neural development (Meyer and Birchmeier, 1995; Gassmann *et al.*, 1995; Lee *et al.*, 1995). A great deal of evidence from both experimental systems and human patients also points to the involvement of HER2 in malignant transformation. In certain tumors, it has been found that HER2 can be overexpressed up to 100 fold, due to gene amplification (Hynes and Stern, 1994). This finding, coupled with the fact that overexpression of HER2 alone, without the addition of agonist for HER family members, can induce focus formation in cultured fibroblasts (Hudziak *et al.*, 1987; DiFiore *et al.*, 1987b) suggests that overexpression of HER2 is capable of inducing oncogenic activity in the human. In addition, overexpression of HER2/*neu* in PC-3 prostate cancer cells has been shown to result in an increased incidence of metastasis after orthotopic introduction (Zhau *et al.*, 1996). Whereas amplification of the gene encoding HER2 is found in 10–30% of breast, ovarian, and gastric tumors (Hynes and Stern, 1994), tumors of the lung, mesenchyme, bladder, and esophagus contain high levels of HER2 protein but no gene amplification, suggesting that both transcriptional and posttranscriptional mechanisms are responsible for increased HER2 levels (Kraus *et al.*, 1987; Hynes *et al.*, 1989; King *et al.*, 1989; Kameda *et al.*, 1990).

HER2 is apparently involved in the genesis of many types of human tumors, but its role has been most well-characterized in breast cancer. Increased levels of HER2 protein appear to correlate with poor patient prognosis (Slamon *et al.*, 1987, 1989; Paik *et al.*, 1990; Gusterson *et al.*, 1992) and a loss of responsiveness to the antiestrogen, tamoxifen (Nicholson *et al.*, 1990; Wright *et al.*, 1992; Klijn *et al.*, 1993). In transgenic mouse models, HER2/*neu* was demonstrated to induce mammary tumors when expression was targeted to the mammary gland by the use of the murine mammary tumor virus promotor (Muthuswamy *et al.*, 1994). These HER2/*neu* tumors contain increased levels of c-Src and c-Yes kinase activity as compared to normal, surrounding tissue (Muthuswamy *et al.*, 1994; Muthuswamy and Muller, 1995). Furthermore, c-Src was found to coimmunoprecipitate with HER2/*neu* (Muthuswamy *et al.*, 1994), suggesting that c-Src cooperates with HER2 as well as with HER1 in regulating malignant progression. Because HER2/*neu* is most frequently localized to the primary tumor mass in the murine model and is found in earlier stage *in situ* carcinomas in humans

(van de Vijver *et al.*, 1988; Paik *et al.*, 1990; Lin *et al.*, 1992; Barnes *et al.*, 1992), it is speculated that this molecule is involved in earlier stages of breast cancer than is HER1.

G. HER Family Members and Estrogen Receptor Interactions

Increasingly compelling data are accumulating that point to interactions among the estrogen receptor (ER), HER1, HER2, and c-Src as being major factors in the development of human breast cancer. The ER is a steroid hormone receptor of 67 kDa that dimerizes and becomes activated as a transcription factor on binding of estrogen (Mangelsdorf *et al.*, 1995). Functional domains of the ER include an amino-terminal A/B region, which is responsible for ligand-independent transcriptional activation; a central DNA-binding domain; and a carboxy-terminal E/F hormone-binding domain, which is responsible for estradiol-induced transcription (Tsai and O'Malley, 1994; Beato *et al.*, 1995). In addition to the well-characterized ER α isoform, a β isoform, which has differing transcriptional properties and expression patterns, has been discovered (Paech *et al.*, 1997).

Loss of ER responsiveness in human breast tumors correlates with overexpression of HER1 and with a poorer patient prognosis (Fitzpatrick *et al.*, 1984; Sainsbury *et al.*, 1985; Davidson *et al.*, 1987; Nicholson *et al.*, 1988). The mechanism by which a breast tumor cell loses responsiveness to estrogen is unclear, but this event may be regulated in part by interactions with HER family members and/or c-Src. Cross-talk between growth factor receptor tyrosine kinases and the ER was first demonstrated by Ignar-Trowbridge and co-workers (1992, 1993), who showed that treatment of cells with EGF activates the transcriptional activity of the ER and that this effect is dependent on the amino-terminal A/B domain of the ER. The ER also appears to have the ability to affect expression of the EGF receptor. In ER-positive breast cancer cells, estradiol treatment increases HER1 mRNA levels (Yarden *et al.*, 1996). This effect may be directly mediated by the ER, because the HER1 promoter has sequences that share loose homology with the estrogen response element (ERE) and can bind human ER (Yarden *et al.*, 1996).

Conversely, HER1 can affect ER expression. Overexpression of TGF- α in the ER-positive ZR75-1 breast cancer cell line, along with prolonged treatment of these cells with antiestrogens, results in loss of the ER, whereas treatment of parental ZR75-1 cells with antiestrogens alone has little effect (Clarke *et al.*, 1989; Agthoven *et al.*, 1992). These results are interpreted to mean that continual and concomitant stimulation of HER1 and ER can cause a reduction in ER expression. In this regard, it is through that expres-

sion of HER1 and ER and mutually exclusive, because most breast tumors that overexpress HER1 lack functional ER (Fitzpatrick *et al.*, 1984; Sainsbury *et al.*, 1985; Davidson *et al.*, 1987; Nicholson *et al.*, 1988). Because breast tumors that do not show this inverse expression tend to be HER1/ER positive rather than HER1/ER negative, it has been suggested that overexpression of HER1 precedes loss of the ER (Koenders *et al.*, 1991; Dittadi *et al.*, 1993; Chrysogelos and Dickson, 1994).

It is unclear how overexpression or activation of HER1 leads to loss of ER expression. One possible mechanism may involve signaling to MAP kinase. HER1 activation results in the phosphorylation of the ER on Ser-118, a phosphorylation that is required for hormone-independent transcriptional activity of the ER (Kato *et al.*, 1995). Ser-118 is also thought to be a target for MAP kinase, because studies using dominant negative Ras and MEK demonstrated a loss of this phosphorylation concomitantly with a loss of EGF-dependent transcriptional activation (Bunone *et al.*, 1996). Autocrine stimulatory loops involving TGF- α and HER1 are known to exist in breast cancer, thus it is speculated that the continued stimulation of the ER via the HER1/MAP kinase pathway leads to its down-regulation and eventual loss.

Estradiol is also known to induce phosphorylation of the ER (Auricchio *et al.*, 1987). In addition, Arnold *et al.* (1995a) reported that the ER is basally phosphorylated on Y537 *in vivo*. The role of the Y537 phosphorylation is controversial. Early studies showed that tyrosine phosphorylation of the ER activates its hormone-binding activity (Migliaccio *et al.*, 1989) and that phosphorylation of Y537 is required for binding of the ER to the ERE (Arnold and Notides, 1995; Arnold *et al.*, 1995b). Further *in vitro* studies demonstrated that c-Src is able to phosphorylate Y537 and that this phosphorylation is necessary for homodimerization of the ER and for binding of estradiol (Arnold *et al.*, 1995a, 1997). In agreement with these findings, Castoria *et al.* (1996) reported that a non-hormone-binding form of the ER found in mammary tumors can be converted to a hormone-binding form by *in vitro* phosphorylation with a calcium/calmodulin-regulated kinase, which is thought to be a c-Src family member. However, additional studies in which Y537 was mutated to various amino acids suggest that phosphorylation of Y537 per se is unnecessary for estradiol-mediated activation of the ER but may be important in ligand-independent (i.e., growth factor-mediated) activation (Weis *et al.*, 1996; Lazennec *et al.*, 1997).

Although c-Src is capable of phosphorylating the ER, the ER may also influence c-Src activity. Estradiol treatment has been shown to increase c-Src tyrosine phosphorylation and kinase activity in MCF7 breast cancer cells (Migliaccio *et al.*, 1993, 1996) and to stimulate kinase activity of c-Src and its related family member, c-Yes, in colon carcinoma cells (Di Domenico *et al.*, 1996).

Ligand-independent down-regulation of the ER may also be mediated by

HER2 signaling pathways. Pietras *et al.* (1995) showed that overexpression of HER2 in MCF7 cells leads to estrogen-independent growth and ERE transcriptional activation. Furthermore, treatment of these cells with HRG, which stimulates HER2-dependent signaling via HER2/3 heterodimers, induces tyrosine phosphorylation and down-regulation of the ER. Other investigators have shown that HRG treatment inhibits the expression of ER in ER-positive breast cancer cells and can revert the estradiol-mediated decrease in HER2 expression (Grunt *et al.*, 1995). Taken together, these results suggest that in experimental cell systems, HER2 and the ER are expressed in a mutually exclusive manner. However, in human breast tumors, the situation is less clear, with some reports indicating an inverse correlation between HER2 and ER expression (Adnane *et al.*, 1989; Borg *et al.*, 1990) and others indicating no such correlation (Slamon *et al.*, 1989; Bacus *et al.*, 1996).

III. c-Src AND c-Src FAMILY MEMBERS IN HUMAN CANCERS

A. c-Src Structure and Mechanisms of Regulation

c-Src is the cellular, nontransforming homolog of v-Src, the oncoprotein encoded by the chicken retrovirus, Rous sarcoma virus. c-Src is a 60-kDa tyrosine kinase that is composed of six domains: an N-terminal membrane-association domain, a "Unique" domain, SH3 and SH2 domains, a catalytic domain, and a negative regulatory domain (Fig. 2, see color plate). Although c-Src is cytosolic, it localizes to intracellular membranes, including the plasma membrane and membranes of endosomes and secretory vesicles within the cytosol (Parsons and Creutz, 1986; Kaplan *et al.*, 1992; Resh, 1994). It is tethered to these membranes by the combined action of an N-terminal, covalently linked myristate moiety, salt bridges between basic amino acids in the N terminus and phosphates of the lipid backbone, and noncovalent interactions with integral or associated membrane proteins (Resh, 1994). Membrane localization of c-Src is required for its ability to participate in growth factor receptor-mediated signaling in normal cells (Wilson *et al.*, 1989). The function of the Unique domain is not well-defined. However, based on the fact that it exhibits the greatest sequence divergence among family members of all the domains (Brown and Cooper, 1996), it is speculated to specify protein-protein interactions that are unique to individual Src family members. The SH3 and SH2 domains mediate the binding of c-Src with other signaling proteins through proline-rich or phosphotyrosine-containing regions on target proteins, respectively (Pawson and Schlessinger, 1993). The major regulatory region of the enzyme is a short domain at the extreme C terminus of

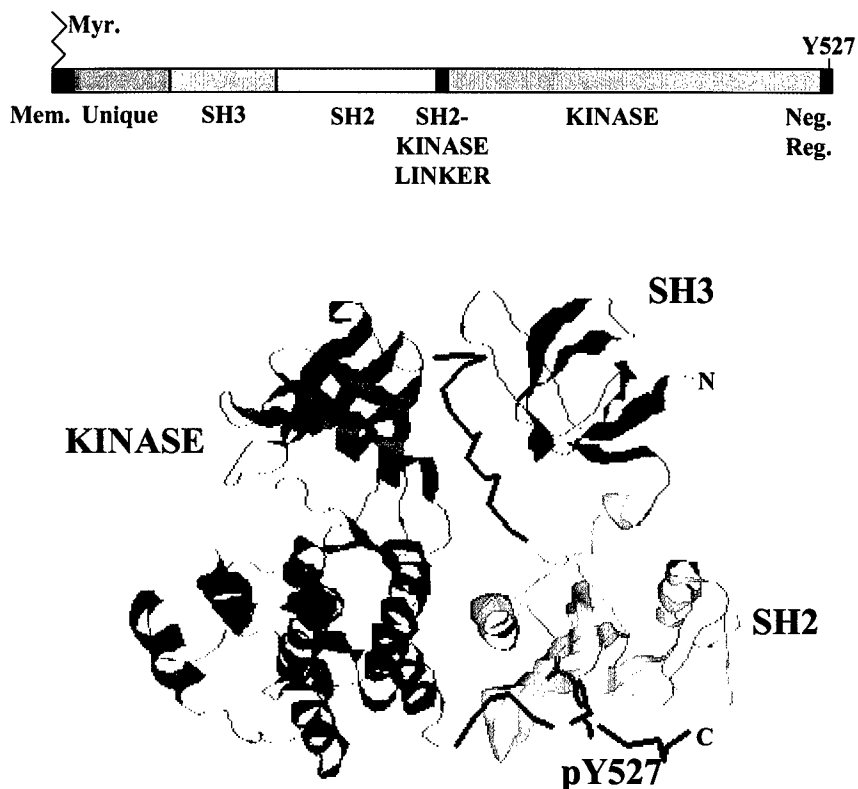


Fig. 2 Structure of c-Src. c-Src is the prototype of a large family of cytoplasmic tyrosine kinases that associate with cellular membranes through lipid modifications at their N termini. As a linear molecule, the relationship between the various domains can be seen: an N-terminal membrane association domain that contains the site of myristylation, a Unique domain that exhibits the widest sequence divergence among family members of any of the domains, an Src-homology-3 (SH3) domain that binds poly(proline) motifs on target molecules, an Src-homology-2 (SH2) domain that binds phosphotyrosine residues on target molecules, an SH2/kinase linker, the catalytic domain, and the negative regulatory domain that contains the predominant site of tyrosine phosphorylation on the inactive molecule (Y527 in chicken, Y531 in human). The three-dimensional orientation of the molecule, lacking the membrane-association and Unique domains, is depicted as a ribbon diagram. Reprinted with permission from *Nature*, Xu *et al.* (1997), and from Michael Eck (configured from the atomic coordinates provided on the Web). Copyright 1997 Macmillan Magazines Limited. The enzymatic activity of c-Src is regulated by the coordinated effects of target proteins binding to or covalent, post-translational modifications of the SH3, SH2, and negative regulatory domains on the catalytic domain, as described in the text.

the molecule, which harbors a Tyr residue that becomes phosphorylated (Y530 in human c-Src; Y527 in chicken c-Src) by a C-terminal Src kinase, CSK (Okada *et al.*, 1991). Phosphorylated Y527/530 (pY527) is capable of binding its own SH2 domain in a manner that inhibits kinase activity without physically blocking the catalytic site, as shown in Fig. 2 (Yamaguchi and Hendrickson, 1996; Sicheri *et al.*, 1997; Xu *et al.*, 1997).

Binding of tyrosine-phosphorylated cellular proteins to the SH2 domain is thought to destabilize the intramolecular pY527/SH2 domain interaction and induce a conformational change that results in enzymatic activation. Structural studies have revealed that the SH2 and SH3 domains collaborate in their binding of respective protein partners, thereby cooperatively influencing the activity of the enzyme (Eck *et al.*, 1994). Furthermore, crystallographic analysis has shown that sequences just N terminal to the catalytic domain (termed the SH2-kinase linker) comprises a loop structure that functions as a "pseudo" SH3 binding site (Yamaguchi and Hendrickson, 1996; Sicheri *et al.*, 1997; Xu *et al.*, 1997). Together, the intramolecular phosphotyrosine/SH2 and linker/SH3 interactions direct a conformation that presses the linker against the backbone of the catalytic domain and renders the protein inactive. As with the SH2 domain, binding of signaling proteins to the SH3 domain is thought to release the constraints of the linker/SH3 interaction on the kinase domain, resulting in activation of catalytic activity.

Mutation of Y527 to F or deletion of the C-terminal regulatory domain (as in v-Src) results in a constitutively active protein that phosphorylates target proteins in an unregulated fashion and induces cellular transformation and oncogenesis (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwnicka-Worms *et al.*, 1987; Reynolds *et al.*, 1987). In normal cells, c-Src is nononcogenic or only weakly so, even when it is overexpressed (Shalloway *et al.*, 1984; Luttrell *et al.*, 1988). However, under certain conditions (growth factor stimulation or translocation; outlined below), the enzyme can become activated, either via dephosphorylation of pY527 or by binding of signaling proteins to the N-terminal half of the protein. Activation is most frequently a transient event, and c-Src, in contrast to v-Src, is thought to respond to negative control by rephosphorylation of Y527 or by the release of binding proteins and the resumption of intramolecular interactions. It has been the conjecture of many investigators that the transient nature of c-Src activation often prevents its detection. In fact, the possibility exists that little or no activation above basal levels is necessary for catalysis, if the substrate is properly positioned near the catalytic cleft. Thus, another "regulator" of c-Src activity may well be its intracellular localization and, at a finer level, its appropriate juxtaposition to substrate within a signaling complex. Identification of c-Src substrates and proteins that bind its SH2 and SH3 domains is now critical for further understanding of the role c-Src and its family members play in biological processes.

The majority of the studies leading to the above model have been conducted in animal tissue culture systems and are just now being applied to the study of c-Src in human tumors. In the following section evidence is presented for the involvement of c-Src in the genesis of human tumors, with particular emphasis on its putative role in colon, breast, lung, and myeloid tumors.

B. Evidence for the Involvement of c-Src in Human Cancers

Like the RTKs, many lines of evidence are suggestive of a role for c-Src in the genesis and progression of multiple types of human cancer. This evidence is both genetic and biochemical in nature and has been generated by studies of cultured tumor cell lines and surgically generated tumor tissue. Together these studies have implicated c-Src as an etiological agent for the development of neuroblastomas, myeloproliferative disorders (including myeloid leukemia), and carcinomas of the colon, breast, lung, esophagus, skin, parotid, cervix, and gastric tissues. Interestingly, although alterations of c-Src have been described at both the gene and protein levels in various cancer tissues, the changes are quite variable and include both increases and decreases in gene copy number and in protein levels and specific enzyme activities. Taken at face value, these findings suggest multiple ways in which c-Src can contribute to the oncogenic process, both as a dominantly acting oncogenic protein and as a negatively acting tumor suppressor. However, the multitude of changes could also reflect fortuitous alterations that do not contribute to the ultimate malignant phenotype. There may also be technical reasons for the variability in the findings, such as the different probes used for genetic analysis and the different antibodies and cell extraction conditions used for biochemical analysis. It is clear that further work needs to be done to clarify these issues and attempts made to minimize technical problems. Of particular importance to future studies will be the development and characterization of good animal and tissue culture models to test the hypotheses derived from analyses of human tumor tissues, whereby the contribution of individual genes or proteins can be evaluated for their oncogenic potential against a normal cell background rather than against a heterogeneous background of unknown numbers and types of genetic alterations that occur in every human tumor.

1. GENETIC EVIDENCE

With the identification of the first protooncogenes came a plethora of studies examining the genomic content of multiple human tumors for deletions, amplifications, and diverse rearrangements in chromosomes containing pro-

tooncogenes. For the most part, these studies identified few if any gross changes in the c-Src gene, which maps to the q arm of chromosome 20. Furthermore, gene expression studies, employing a variety of techniques to measure steady-state levels and newly synthesized mRNA have also revealed few changes in c-Src-specific mRNA (Bishop, 1983; Slamon *et al.*, 1984). These findings led many investigators to conclude that c-Src played a minor (if any) role in the genesis of human tumors. Not until researchers began examining protein levels and specific enzyme activities did evidence for the involvement of c-Src begin to emerge.

However, there were a few exceptions to the general rule described above, and one in particular is noteworthy. Four groups have identified a deletion of 16–21 cM in the long arm of chromosome 20 [del(20q)] as a recurring, nonrandom abnormality in malignant myeloid disorders, including non-lymphocytic leukemia and polycythemia (Simpson, 1988; Roulston *et al.*, 1993; Hollings, 1994; Asimakopoulous *et al.*, 1994). This deletion maps between 20q11.2 and 20q13.3, a region that encodes the c-Src protooncogene (Hollings, 1994). The notion that deletion of a chromosomal region is signatory for a tumor suppressor gene suggests that, if c-Src is a critical gene in this deletion, it behaves as a negative regulator of cell growth, not as a dominant oncogene, as is commonly believed. That c-Src may have some tumor suppressor-like characteristics in myeloid cells is supported by the finding of several groups (Barnekow and Gessler, 1986; Gee *et al.*, 1986) that c-Src expression levels increase during myeloid differentiation. If c-Src plays a critical role in promoting differentiation and maintaining the postmitotic state, then loss of such an activity might permit cells to once again acquire proliferative activity—the hallmark of a tumor suppressor.

2. BIOCHEMICAL EVIDENCE

By far the bulk of evidence supporting a role for c-Src in the development of human tumors comes from biochemical studies, wherein the levels of c-Src protein and tyrosine kinase activity have been examined in hundreds of human tumors and compared to normal tissue controls. As will be discussed in more detail below, in some tumor specimens, high enzymatic activity is accompanied by high protein level, yielding little or no change in specific activities, whereas in others, protein levels are only slightly or modestly elevated, and the specific activity of the enzyme is increased. In yet other examples, high protein levels are accompanied by low enzymatic activity. However, the overall conclusion is that in a very high percentage (>50% and approaching 100% in some studies) of human tumors of many different tissue types, c-Src activity is altered (usually elevated) and that this alteration occurs in early to middle stages of tumor progression and is maintained or increased throughout progression to metastasis.

These findings raise questions as to the mechanism of c-Src activation and the mechanisms by which protein levels are elevated (especially in light of the few instances of increases in c-Src-specific mRNA production). The consensus at the present time is that changes in c-Src specific activity in human tumors are due to posttranslational events and not to mutations of the gene. Using RNase protection and restriction fragment-length polymorphism assays to detect activating mutations of c-Src in a spectrum of human tumors, Wang *et al.* (1991) were unable to detect mutations at codons known to contribute to the oncogenicity of v-Src and c-Src (namely, codons 98, 381, 444, and 530 in the human c-Src sequence). These findings led the investigators to conclude that mutational activation is not the mechanism of enhancement of c-Src-specific kinase activity. On the other hand, DeSeau *et al.* (1987) described differential activation of c-Src in normal colon cells versus colonic tumor cells depending on the conditions of extract preparation, i.e., whether the lysis buffers contained the proteins tyrosine phosphatase inhibitor, vanadate, and/or high concentrations of ionic and nonionic detergents. From these results, one could deduce that tyrosine phosphorylation of c-Src or other cellular proteins and protein/protein interactions play a role in regulating not only c-Src activity but also its stability and abundance. Indeed, structural studies on the c-Src molecule described above would support this notion. However, so as not to think that the issue is resolved, studies by Watanabe *et al.* (1995) indicate that in 18 cancer cell lines, elevated activities of c-Src and c-Yes (a Src-related family member) are accompanied by correspondingly elevated levels of C-terminal Src kinase, the protein that phosphorylates Y530 in human c-Src and negatively regulates c-Src kinase activity. These findings suggest that CSK may not have an antioncogenic role to play in tumor progression or that dephosphorylation of Y530 is not required for activation of c-Src.

Here the focus is on three different carcinomas—colon, breast, and lung—for which substantial amounts of data are accumulating to indicate a role for c-Src in their development. That these represent three of the four most common forms of cancer in adults (prostate cancer being the fourth) suggests that c-Src may be a more formidable player in tumorigenesis than had previously been appreciated.

3. COLON CANCER

Utilizing c-Src-specific antibodies and an immune complex-based tyrosine kinase assay, a number of investigators have reported that c-Src-specific tyrosine kinase activity (total activity relative to total c-Src protein in an immune complex) is elevated in colon cancer. In panels of colon cancers examined by Rosen *et al.* (1986), Bolen *et al.* (1987a,b), and Cartwright *et al.*

(1989), c-Src was found to exhibit elevated kinase activity, ranging from ~2- to 40-fold above that found in normal colon tissues or cultures of normal colon mucosal cells. In some cases this increase could be accounted for by increases in protein levels, but in other instances it could not, indicating an increase in specific kinase activity. These results suggest that either elevation in c-Src protein and/or activation of c-Src may contribute to the genesis of human colon tumors. Indeed, additional studies by Lundy *et al.* (1988) and Cartwright *et al.* (1990, 1994) demonstrated increased kinase activity in premalignant epithelia of ulcerative colitis and in early-stage colonic polyps as compared to adjacent normal mucosa. In the latter study, activity was highest in malignant polyps and in >2-cm benign polyps that contained villous structure and severe dysplasia. Thus, c-Src activity is found to be elevated in early stages of colon cancer and this elevation is associated with those polyps that are at greatest risk for developing cancer. Talamonti *et al.* (1993) also demonstrated incremental increases in c-Src activity and protein level as the tumors progressed, with the greatest increases seen in metastatic lesions. Increases in specific kinase activity were also observed, with liver metastases exhibiting an average increase of 2.2-fold over normal mucosa, whereas extrahepatic metastases demonstrated an average 12.7-fold increase. These results support the idea that c-Src may play multiple roles in tumor progression.

A number of studies have been done to determine if c-Src indeed plays a causal role in tumor development. Herbimycin A, an inhibitor of Src family kinases, was shown to inhibit the growth in monolayer of seven colon tumor cell lines as compared to one cell line from normal colonic mucosa, CCL239 (Garcia *et al.*, 1991). In another study, blockage of the myristylation modification of Src family members in a panel of human colon adenocarcinoma tumor cell lines by *N*-fatty acyl glycinal compounds was shown to prevent localization of c-Src to the plasma membrane and to depress colony formation of these cell lines in soft agar and cell proliferation assays (Shoji *et al.*, 1990). Tumor necrosis factor (TNF- α -mediated growth inhibition of human colorectal carcinoma cell lines was accompanied by a reduction in the activity of s-Src (Novotny-Smith and Gallick, 1992). And last, using an antisense expression vector specific for c-Src, Staley *et al.* (1997) demonstrated that expression of c-Src antisense in HT29 human colon adenocarcinoma cells resulted in slower proliferation and slower growing tumors in nude mice as compared to the parental control. Together, these studies are consistent with a causative role for c-Src in colon cancer progression.

How could c-Src be functioning to promote progression of colonic tumors? Using an *in vitro* progression model based on the PC/AA premalignant colonic adenoma cell line, Brunton *et al.* (1997) demonstrated that in the conversion from adenoma to carcinoma, levels of both the EGF receptor and FAK protein increased, while the expression and activity of c-Src were

unaltered. However, EGF induced motility in the carcinoma cells, but not in the adenoma cells, and this increase was accompanied by an EGF-induced increase in c-Src kinase activity, relocalization of c-Src to the cell periphery, and phosphorylation of FAK. The authors interpret these findings to indicate that c-Src is not the driving force for tumor progression, but cooperates with other molecules (such as EGFR and FAK) in the process. Other investigators have observed that adhesion of HT29 human colon carcinoma cells to E-selectin results in a decrease in c-Src activity (Soltesz *et al.*, 1997), suggesting that, on release from substratum restrictions, c-Src activity is restored or elevated. In a related study, Empereur *et al.* (1997) generated evidence for cooperativity between c-Src and HGF/SF in developing invasive properties of the PC/AA cell line. Specifically, introduction of activated c-Src or polyoma middle-T antigen (which requires c-Src for oncogenic activity) into the adenoma PC/AA cell line induced conversion of the adenoma to carcinoma, overexpression of the HGF receptor, and an invasive capacity in the presence of HGF. Thus, current evidence suggests that one mechanism by which c-Src promotes colonic tumor progression is by cooperating with components of the cell adhesion/motility machinery. Similar conclusions were reached by Mao *et al.* (1997), who demonstrated activation of c-Src in response to EGF or HGF treatment of human colon cancer cells with high metastatic potential.

4. BREAST CANCER

As with colon cancer, a number of early investigations reported elevated c-Src activity in human breast cancers (Jacobs and Rubsamen, 1983; Rosen *et al.*, 1986; Lehrer *et al.*, 1989). In several reports the elevation in activity was not accompanied by elevated levels of c-Src protein, suggesting an activation of the protein. However, Koster *et al.* (1991), using a screening method based on *in vitro* synthesis of cDNA copied from total cellular RNA of tumor tissue, found that 25–30% of the analyzed tumors showed significant elevations in expression of several protooncogenes, including c-Src. Using immune complex kinase assays, immunoblotting, and immunohistochemical approaches, Verbeek *et al.* (1996) and Biscardi *et al.* (1998a) demonstrated that increases in c-Src kinase activity are almost invariably accompanied by increases in c-Src protein levels and little if any change in specific kinase activity. Interestingly, the immunohistochemical studies of Verbeek *et al.* (1996) showed that in malignant cells, the majority of c-Src appeared to be concentrated around the nucleus, whereas in normal cells, it is distributed more evenly in the cytoplasm. The discrepancies between the more recent data and the earlier data may reflect changes in the quality of the antibodies and the more quantitative analyses performed in the recent studies. In total, the current evidence indicates that few “activations” of c-

Src occur in breast tumor cells; rather, elevations in protein levels appear to be the major cause of the increases in c-Src kinase activity. In a recent study involving 72 breast cell lines and tumor biopsies, tyrosine kinase activity was found to be elevated in 100% of the samples, as compared to normal tissue controls, and c-Src tyrosine kinase accounted for 70% of the total cytosolic activity (Ottenhoff-Kalff *et al.*, 1992). The same group performing that study had previously found that the level of cytosolic protein tyrosine kinase activity parallels the malignancy in breast tumors (Hennipman *et al.*, 1989) and that the majority of this activity is precipitated by anti-c-Src antibodies. These results provide compelling correlative evidence that c-Src plays a key role in the development of breast cancer. In agreement with this conclusion, Lehrer *et al.* (1989) and Koster *et al.* (1991) also note that elevated c-Src kinase activity is most frequently found in tumors that are progesterone receptor negative. Because loss of progesterone receptor is a histochemical marker for later stage tumors, c-Src activity appears to increase as the tumor progresses in severity.

To directly assess the effect of mammary gland-specific expression of c-Src, Webster *et al.* (1995) established transgenic mice that carried a constitutively activated form of c-Src under the transcriptional control of the murine mammary tumor virus long terminal repeat. Female transgenic mice exhibited a lactation defect and frequently developed mammary epithelial hyperplasias, which occasionally progressed to frank neoplasias. The authors interpret these results to mean that expression of activated c-Src in the mammary gland is not sufficient for induction of mammary tumors—that some other event must take place for frank neoplasias to occur. That c-Src can play more than a bystander role in tumor development, however, was demonstrated by the experiments of Guy *et al.*, (1994), wherein mice transgenic for the polyoma virus middle-T antigen under the control of the murine mammary tumor virus long terminal repeat developed tumors when in a genetic background positive for c-Src, but not when in a background null for c-Src. Similar results were obtained by Amini *et al.* (1986a), who used c-Src antisense expression vectors to demonstrate that c-Src is required for transformation of rat FR3T3 cells by polyoma middle-T antigen in tissue culture. Together, these studies indicate that c-Src is necessary but not sufficient for tumor development in the mammary gland.

5. LUNG CANCER

Lung cancer is the leading cause of cancer death in the United States. Small cell lung cancer (SCLC) accounts for 20–25% of all bronchogenic carcinoma and is associated with the poorest 5-year survival of all histologic types. c-Src expression was found to be elevated in 60% of all lung cancers (Mazurenko *et al.*, 1991b), when biopsy material of tumors, metastases, and

"normal" surrounding tissues from patients with different histological types of stomach and lung cancer, melanoma, and other malignancies were analyzed by immunoblotting and immunohistochemistry. A breakdown of the lung histologic types exhibiting increased c-Src expression revealed that c-Src protein was elevated in SCLC and atypical carcinoid tumors, as well as in non-small-cell tumors, such as adenocarcinoma, bronchoalveolar, and squamous cell lung cancer (Mazurenko *et al.*, 1991a). In these studies no analysis of c-Src kinase activity was reported. Somewhat contrasting results were reported by authors of a study in which 60 human cell lines used by the National Cancer Institute for the random screening of potential anticancer drugs were analyzed for c-Src kinase activity. In this study SCLC-derived cell lines had a low activity, whereas non-small-cell lung tumors exhibited activity that was greater than that observed in colon cancer cells, which are considered to have high c-Src activity (Budde *et al.*, 1994). The findings from these studies are strongly supportive of other investigations, concluding that c-Src is frequently overexpressed in SCLC and other types of lung cancer (Cook *et al.*, 1993).

6. OTHER CANCERS

Many other tumor types exhibit elevations in c-Src kinase activity or protein/mRNA levels, including neuroblastomas (Bjelfman *et al.*, 1990) and carcinomas of the esophagus (Jankowski *et al.*, 1992; Kumble *et al.*, 1997), gastric tract (Takekura *et al.*, 1990), parotid gland (Bu *et al.*, 1996), ovary (Budde *et al.*, 1994), and skin (Kim *et al.*, 1991). With regard to skin cancers, a study carried out in a mouse model of epidermal tumor promotion described activation of erbB2 and c-Src in phorbol ester-treated mouse skin as a possible mechanism by which phorbol esters promote skin tumors in mice. Activation of erbB2 and c-Src kinase is also observed in the epidermis of TGF α transgenic mice, where expression of human TGF α was targeted to basal keratinocytes (Xian *et al.*, 1997). In cervical cancer, evidence is beginning to emerge for cis activation of cellular protooncogenes (including c-Src) by integration of human papillomavirus DNA into the genome of cervical epidermal cells (Durst *et al.*, 1987). In tissue culture studies using primary hamster embryo cells, infection with other DNA tumor viruses, such as SV40, adenovirus, or bovine papillomavirus, also results in increases in the specific activity of c-Src (Amini *et al.*, 1986b).

C. c-Src Family Members and Human Cancers

c-Src is the prototype for a family of nonreceptor protein tyrosine kinases, for which novel members are regularly being identified. Current members

include c-Src, Fyn, c-Yes, Lck, Hck, Lyn, c-Fgr, Blk, and Yrk (Brickell, 1992; Sudol *et al.*, 1993; Brown and Cooper, 1996). All members have the same overall structure and minimally contain Unique, SH3, SH2, and kinase domains. The greatest sequence divergence occurs in the Unique domain, thus its name. Not all members are linked to lipids at the N terminus, nor are all negatively regulated by a C-terminal domain that includes the Tyr-530 homolog of human c-Src. c-Fgr, Lck, Hck, and Blk are expressed predominantly in cells of hematopoietic lineage, whereas c-Src, c-Yes, Fyn, Lyn, and Yrk are more ubiquitous. All members have been implicated in various signal transduction pathways, and with the mounting evidence for involvement of c-Src in the genesis of multiple human cancers, the question arises as to whether close relatives of c-Src may also be implicated in these diseases. If so, there are other questions that warrant investigation: Is more than one family member involved in the genesis of the same tumor type? Do c-Src family members fulfill overlapping or unique functions in promoting tumor formation and progression? Are there members of this family that are expressed exclusively in tumors as compared to normal tissue? A review of the literature reveals a paucity of information with regard to any of these issues. It is not clear whether this paucity reflects the unavailability of useful and appropriate reagents to investigate the questions, or whether studies have been conducted and few have uncovered evidence for c-Src family member involvement. Although the following description is not meant to be comprehensive, it does suggest that family members in addition to c-Src may be involved in the genesis of human and certain animal tumors.

1. GENETIC EVIDENCE

In humans, sequences related to the human c-Yes gene were found to be amplified in a single primary gastric cancer out of 22 cases that were examined (Seki *et al.*, 1985). The sequences were amplified four- to fivefold, but normal stomach tissue adjacent to the tumor tissue in the same patient showed no amplification. In the dog, a protooncogene related to the human c-Yes gene was detected as restriction fragment-length polymorphisms (RFLPs) in a Southern blot analysis of genomic DNA from six canine primary mammary tumors in a screen employing seven protooncogene probes. These RFLPs were 0.1 to 1.0 kb shorter than the normal gene, suggesting the occurrence of chromosomal rearrangements and possible deregulation of gene expression, leading to tumorigenesis (Miyoshi *et al.*, 1991). Melanoma formation in the fish *Xiphophorus* is a genetic model for the function of tyrosine kinases in tumor development. In malignant melanomas from these fish, elevated levels of c-Yes and Fyn activity have been detected as compared to normal tissue (Hannig *et al.*, 1991). Fyn has also been found to coprecipitate with the *Xiphophorus* melanoma receptor kinase (Xmrk), the molecule

that is responsible for the formation of hereditary malignant melanoma in this lower vertebrate (Wellbrook *et al.*, 1995). These results suggest that Xmrk may function at least in part through Fyn in melanoma formation.

2. BIOCHEMICAL EVIDENCE

In studies similar to those conducted for c-Src, evidence for the involvement of other c-Src family members in the etiology of human cancers is emerging, but at a much slower pace than that for c-Src. Elevated c-Yes tyrosine kinase activity has been detected in premalignant lesions of the colon that are at greatest risk for developing cancer (Pena *et al.*, 1995). In this study, the activity of c-Yes in such adenomas was 12- to 14-fold greater than activity in adjacent normal mucosa. Similar results were obtained when mRNA levels of nine protooncogenes in colonic tissue from patients with inflammatory bowel disease (IBD) were measured. The steady-state level of c-Yes-encoded mRNA was considerably higher in IBD patients resected for colon cancer than in patients resected for active chronic IBD or in controls (Alexander *et al.*, 1996). These results suggest that expression of this gene may be a marker for development of colon cancer in IBD. Finally, in rodents, the action of the transforming proteins of mouse and hamster polyomaviruses (middle-T antigens) is mediated in part through c-Src family kinases, with preferential action of hamster T antigen for Fyn (Brizuela *et al.*, 1995).

c-Src family members have also been implicated in the genesis of diseases involving Epstein-Barr Virus (EBV), such as Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal cancer. All of these diseases involve abnormal proliferation of B cells. EBV encodes two transformation-associated proteins, LMP1 and LMP2, that are integral membrane proteins. LMP2 mRNA is the only EBV-specific message detected in B lymphocytes from individuals harboring EBV latent infections. LMP2 protein also associates with c-Src family tyrosine kinases, LMP1, and other unidentified proteins, suggesting that the association of these two EBV-encoded membrane proteins could create a macromolecular complex mediating constitutive B lymphocyte activation through normal cell signal transduction pathways (Longnecker, 1994).

In human malignant melanoma and other cancers, aberrant expression of basic fibroblast growth factor (bFGF) causes constitutive autocrine activation of its cognate receptor and autonomous growth of tumor cells in culture (see above). Expression of a dominant-negative mutant of the FGF receptor (lacking the kinase domain) was found to suppress tumor formation in nude mice and markedly reduce c-Src family kinase activity in melanoma cells (Yayon *et al.*, 1997). Together these studies suggest that c-Src family kinases play an important role in maintenance and/or progression of malignant melanoma.

D. Nonreceptor Tyrosine Kinases Related to c-Src Family Members and Human Cancers

Several new nonreceptor tyrosine kinases have been isolated from human breast cancer cells. A cDNA encoding a 54-kDa phosphoprotein, called Rak, was cloned from human breast cancer cells (Cance *et al.*, 1994). This protein shares 51% identity with c-Src and contains SH3, SH2, kinase, and negative regulatory domains. However, it has some properties that are distinct from c-Src, such as its predominant expression in epithelial cells, its lack of a myristylation site, and its almost exclusive localization to the nucleus. However, like c-Src, Rak is overexpressed in subsets of primary human epithelial tumors, suggesting that it may play a role in development of human cancer. Another protein, named Brk (breast tumor kinase), appears to be expressed exclusively in breast tumor tissue as opposed to normal mammary epithelium (Barker *et al.*, 1997). Approximately two-thirds of breast tumors express appreciable levels, and 27% of these overexpress Brk 5- to 40-fold or more. When overexpressed in fibroblasts or mammary epithelial cells, Brk sensitizes cells to the action of EGF and also induces a partial transformed phenotype (Kamalati *et al.*, 1996). These findings suggest that Brk is a functionally important factor in the evolution of breast cancer.

IV. MECHANISMS OF c-Src ACTION

A. Evidence for Involvement of c-Src in Signaling through Receptor Tyrosine Kinases

The elevated levels of c-Src expression and/or activation in a wide spectrum of human tumors suggest that c-Src is contributing in some way to the neoplastic phenotype. That c-Src is overexpressed in many of the same tumors in which specific RTKs are also often overexpressed suggests that the two classes of tyrosine kinases may functionally interact to promote tumorigenesis. Many of the RTKs are oncogenic when overexpressed or inappropriately expressed, as described above. The question that follows is whether c-Src is required for the oncogenic capabilities of overexpressed RTKs, or whether c-Src enhances or contributes to RTK-mediated oncogenesis by any means. This latter question was in part addressed when it was shown that cooverexpression of c-Src with HER1 in a mouse fibroblast model resulted in synergistic increases in tumor volume, as compared to tumors developed by cells overexpressing only one of the pair of kinases (Maa *et al.*, 1995). These results provided direct evidence for the enhancing effect of c-Src on receptor-transforming ability, and suggested that a similar synergism

may be occurring in human tumors that cooverexpress c-Src and the EGFR or other RTKs. Targets of c-Src action can be inferred from an analysis of its known intracellular substrates, which, besides the cell surface receptors, are almost exclusively proteins that regulate actin cytoskeleton dynamics. Thus, c-Src appears to have the capability of affecting both mitogenic growth pathways and morphogenic pathways that influence cell/matrix and cell/cell interactions, motility, invasiveness, and metastasis. Here the focus is on studies that are beginning to reveal the molecular interactions between c-Src and its substrates (as they relate to malignancy) and the effects phosphorylation by c-Src have on their functions. It is becoming clear that c-Src is an obligate partner in mediating mitogenic signaling of at least two RTKs, specifically the PDGF and EGF receptors, and that in the case of EGFR, c-Src mediates tumorigenic signaling as well. This new information, in turn, can be used to design novel diagnostics and therapeutics to interdict the symbiotic relationship between c-Src and the RTKs.

A number of different growth factor receptors that have been shown to associate with or activate c-Src or Src family members were enumerated in Section I. These included receptors for PDGF, CSF-1, HGF/SF, and EGF, as well as HER2. With the exception of the PDGFR and EGFR, little is known about the role of c-Src in signaling through these receptors, other than the fact that c-Src either associates with the receptor or is activated following specific ligand stimulation. Therefore, we focus our discussion on c-Src interactions with the PDGF and EGF receptors. The data implicating c-Src in PDGF-dependent signaling will be briefly summarized, this being the subject of several other reviews. The bulk of our attention will then be focused on the mechanism of interaction between c-Src and EGFR family members.

I. ROLE FOR c-Src IN SIGNALING FROM THE PDGFR

The first evidence that c-Src participates in PDGFR signaling came from the work of Ralston and Bishop (1985), who first observed that c-Src becomes activated on PDGF stimulation. Kypta *et al.* (1990) later demonstrated that c-Fyn and c-Yes are also activated in a PDGF-dependent manner. Activation of c-Src was shown to be accompanied by a translocation of c-Src from the plasma membrane to the cytosol (Walker *et al.*, 1993), a process that may be linked to internalization of the receptor. PDGF stimulation was also shown to stimulate transient association of Src family members with the PDGFR (Kypta *et al.*, 1990). Association between Src family members and the receptor is believed to involve phosphotyrosine-SH2 interactions, because the SH2 domain of c-Fyn is required for binding to the receptor *in vitro* (Twamley *et al.*, 1992) and mutation to phenylalanine of Y579 and Y581 in the juxtamembrane region of the receptor results in a decrease in both PDGF-induced c-Src activation and binding to the receptor *in*

vivo (Mori *et al.*, 1993). These data and results from *in vitro* peptide binding studies (Alonso *et al.*, 1995) suggest that Y579 and Y581 directly mediate binding of Src family members to the PDGFR.

Interaction of c-Src with the PDGFR appears to have consequences for both c-Src and the PDGFR. Hansen *et al.* (1996) have shown that Y934 in the kinase domain of the PDGFR is phosphorylated by c-Src both *in vitro* and *in vivo*. Expression of a receptor harboring a phenylalanine substitution at residue 934 in intact cells results in a decreased mitogenic signal and an increase in chemotaxis and motility, along with enhanced PLC γ tyrosine phosphorylation. These data suggest that phosphorylation of Y934 by c-Src positively regulates mitogenesis, while negatively regulating cell motility, possibly via a PLC γ -mediated pathway. Activation of c-Src by PDGF is also accompanied by the appearance of novel phosphorylations on c-Src, including two serine phosphorylations, S12 and an unidentified S residue (Gould and Hunter, 1988), and one tyrosine phosphorylation, Y138 (Broome and Hunter, 1997). Y138 is located in the SH3 domain of c-Src, and phosphorylation of this residue diminishes the ability of peptide ligands to bind the SH3 domain *in vitro*. Mutation of Y138 or Y133 to phenylalanine or complete deletion of the SH3 domain reduces the mitogenic effect of PDGF (Erpel *et al.*, 1996; Broome and Hunter, 1996). The hypothesis that Src family members are required for PDGF-dependent signaling is supported by the inhibitory effects of kinase-inactive c-Src or an antibody specific for the C-terminal domain of Src family members on PDGF-induced BrdU incorporation into newly synthesized DNA (Twamley-Stein *et al.*, 1993).

2. ROLE OF c-Src IN SIGNALING FROM EGFR

In our laboratory, initial attempts to detect EGF-induced alterations in c-Src kinase activity or physical association between c-Src and the EGFR in a panel of nontransformed avian and rodent cell lines were negative, or yielded inconsistent results (Luttrell *et al.*, 1988). Therefore, a direct test of the involvement of c-Src was undertaken, in which wild-type (wt) and mutational variants of c-Src were overexpressed in C3H10T1/2 mouse fibroblasts, and the effect of overexpression of these variants on EGF-induced [3 H]thymidine incorporation was examined. Overexpression of wt c-Src resulted in a two- to fivefold increase in [3 H]thymidine incorporation above Neo-only controls (Luttrell *et al.*, 1988), whereas overexpression of c-Src harboring inactivating mutations in the kinase, SH2, or myristylation domains resulted not only in a reduction in the enhanced effect of overexpressed wt c-Src but also in a dominant negative effect on endogenous, EGF-induced DNA synthesis (Wilson *et al.*, 1989). These results indicated not only that c-Src is required for mitogenesis stimulated by EGF, but also that c-Src kinase activity, an intact SH2 domain, and membrane association are

necessary to fulfill the role of c-Src in the process. These findings were corroborated by studies in NIH3T3 cells, in which a decrease in EGF-induced BrdU incorporation was observed on microinjection of antibodies to c-Src family members or introduction of a kinase-inactive c-Src cDNA into cells (Roche *et al.*, 1995).

c-Src was also shown to affect EGF-induced tumorigenesis (Maa *et al.*, 1995). In C3H10T1/2 cells, coexpression of c-Src and the HER1 results in synergistic increases in proliferation, colony formation in soft agar, and tumorigenicity in nude mice, as compared to cells overexpressing c-Src or HER1 alone. Furthermore, under conditions of receptor and c-Src overexpression, an EGF-inducible complex between the proteins can be detected. Enhanced tumor growth correlates with the ability of c-Src to associate stably with the receptor, the appearance of two novel tyrosine phosphorylation sites on the receptor, and enhanced phosphorylation of the receptor substrates, Shc and PLC γ . These findings suggest that c-Src association with and phosphorylation of the receptor results in hyperactivation of the receptor and enhanced mitogenic signaling to downstream effectors. Subsequent investigations have revealed that the kinase activity of c-Src is required for the biological synergy between c-Src and overexpressed HER1 (Tice *et al.*, 1998). Kinase-defective c-Src, when expressed in a cell line overexpressing HER1, acts in a dominant negative fashion to inhibit EGF-dependent colony formation in soft agar and tumorigenicity in nude mice. The effects of both wt and kinase-defective c-Src are very striking, with the single wt c-Src or HER1 overexpressors forming barely detectable tumors in nude mice ($<300 \text{ mm}^3$) and the c-Src/HER1 double overexpressor forming large tumors ($\sim 1600 \text{ mm}^3$). In contrast, the HER1/kinase-defective c-Src overexpressors form no palpable tumors. Thus, the extent of tumor inhibition by kinase-defective c-Src is complete in this model system, and the results suggest that the catalytic activity of c-Src may be a fruitful target for human tumor therapy. Interestingly, expression of c-Src variants that bear mutations in either the SH2 or myristylation domains augments, rather than inhibits, tumor formation of cells overexpressing the receptor (D. A. Tice, unpublished). These results are in surprising contrast to those observed when the same c-Src variants are expressed in cells containing normal levels of EGFR (see above and Wilson *et al.*, 1989). The mechanism by which tumor growth is enhanced by these variants is not known.

The mechanism of synergy between wt c-Src and HER1 is beginning to be elucidated. Several groups have now demonstrated an EGF-dependent complex formation between c-Src and the HER1 (Luttrell *et al.*, 1994; Maa *et al.*, 1995; Stover *et al.*, 1995) and an EGF-induced activation of c-Src-specific kinase activity (Oshero and Levitzki, 1994; Oude Weernink *et al.*, 1994). In all instances, these events are seen in cells overexpressing one or both partners, suggesting that the interaction is either transient or low affini-

ity. In addition, there is evidence for phosphorylation of HER1 by c-Src on EGF stimulation. In the C3H10T1/2 murine fibroblasts, two sites of tyrosine phosphorylation on c-Src-associated HER1 have been identified both *in vitro* and *in vivo* as Y845 and Y1101 (Biscardi *et al.*, 1998b). Y845 has also been identified as a c-Src-specific phosphorylation site in A431 cells (Sato *et al.*, 1995) and in MDA-MB-468 breast cancer cells (Biscardi *et al.*, 1998b), whereas two other nonautophosphorylation sites, Y891 and Y920, were identified on the receptor from MCF7 cells (Stover *et al.*, 1995). Tice *et al.* (1998) have shown in 10T1/2 cells that Y845 is the only phosphorylation that is completely dependent on c-Src kinase activity, implicating a direct phosphorylation of the receptor by c-Src at this site, and suggesting that phosphorylation of other c-Src-dependent sites may involve a third component.

Y845 is located in the activation loop of the kinase domain and is highly conserved among all tyrosine kinases, receptor and nonreceptor alike. Its homolog in c-Src is Y416. Phosphorylation at the homologous site in other kinases is required for full enzymatic activation, through ATP and substrate accessibility (Ellis *et al.*, 1986; Fantl *et al.*, 1989; Knighton *et al.*, 1991; van der Geer and Hunter, 1991; Longati *et al.*, 1994; Kato *et al.*, 1994; Russo *et al.*, 1996; Yamaguchi and Hendrickson, 1996; Mohammadi *et al.*, 1996; Hubbard, 1997). In 10T1/2 cells the presence of this phosphorylation on the HER1 correlates with an increase in tyrosine phosphorylation of receptor substrates Shc and PLC γ , and enhanced growth and tumor formation (Maa *et al.*, 1995), consistent with hyperactivation of the receptor. Conversely, the absence of this phosphorylation (in 10T1/2 cells overexpressing receptor and kinase-defective c-Src) correlates with reduced growth and tumor formation. Thus, phosphorylation of Y845 appears to be required for the oncogenic capabilities of the receptor. Interestingly, in all other kinases but HER1, the Y845 homolog is an autophosphorylation site. That Y845 has not been identified as such for the HER1 may be due to the high lability of the phosphorylation (Biscardi *et al.*, 1998b), or to the fact that c-Src appears to be the kinase that phosphorylates it (Tice *et al.*, 1998). Phosphorylation of Y845 also appears to be critical for normal signaling through the receptor. This is supported by recent findings that a Y845F mutation completely ablates EGF or serum-induced DNA synthesis, either in the presence or absence of overexpressed c-Src (Tice *et al.*, 1998). Thus, the ability of c-Src to phosphorylate Y845 is critical for manifestation of both the mitogenic and tumorigenic properties of the receptor.

a. EGF Receptor Internalization

Based on evidence that implicates the actin cytoskeleton as critical for EGFR internalization (Lamaze *et al.*, 1997), and the involvement of c-Src and c-Src substrates in actin dynamics, as well as the localization of c-Src to

membranes of intracellular vesicles (Parsons and Creutz, 1986; Kaplan *et al.*, 1992), it is reasonable to speculate that c-Src enhances EGF-dependent signaling by influencing receptor internalization. One hypothesis is that c-Src enhances mitogenesis and tumorigenesis by inhibiting internalization and prolonging receptor signaling at the plasma membrane. In surprising contrast to this hypothesis, however, it was found in studies of 10T1/2 cells that c-Src overexpression enhances rather than inhibits receptor internalization by increasing steady-state pools of internalized, activated receptors (Ware *et al.*, 1997). Receptor recycling rates are not altered. The kinase activity of c-Src is required for the increase, because overexpression of kinase-deficient c-Src exhibits basal or slightly reduced internalization rates.

How might the increased internalization contribute to the enhanced cell proliferation and tumorigenic potential seen in cells overexpressing wt c-Src? Recent evidence indicates that receptor/SHC/GRB2/SOS complexes are present in endosomes (DiGuglielmo *et al.*, 1994), suggesting that EGF/EGFR complexes continue to signal in the endosomal compartment (Baass *et al.*, 1995; Bevan *et al.*, 1996). Because c-Src overexpression increases the steady-state pool of internalized, activated receptors, c-Src may enhance mitogenic and tumorigenic signaling by promoting the frequency of interactions between receptor complexes in the endosomes and Ras at the plasma membrane.

Although the mechanism by which c-Src affects EGFR internalization is unknown, several possibilities are plausible. First, c-Src may increase the rate of association of the EGFR with components of the endocytic pathway, such as the adaptins (Sorkin and Carpenter, 1993), or Grb2 (Wang and Moran, 1996), which are thought to recruit activated receptors into clathrin-coated pits. Interestingly, Grb2 associates with dynamin (Gout *et al.*, 1993), a GTPase that is critical for the formation and release of the endosome from the plasma membrane. The c-Src SH3 domain is also reported to activate the GTPase activity of dynamin *in vitro* (Herskovits *et al.*, 1993). These considerations suggest that as a second mechanism, overexpression of c-Src could result in the activation or recruitment of a pool of dynamin larger than that in cells expressing normal levels of c-Src.

A third mechanism by which c-Src may affect EGFR internalization is through processes that do not involve clathrin-coated pits, such as through caveolae. Caveolae are small invaginations of the plasma membrane that have been implicated in the transcytosis of macromolecules across capillary endothelial cells, the uptake of small molecules, interactions with actin-based cytoskeleton, and the compartmentalization of certain signaling molecules, including G-protein-coupled receptors, H-Ras and Ras-related GTPases, and members of the Src family of tyrosine kinases (Li *et al.*, 1996a,b). Caveolae are enriched for a specific protein, caveolin, which is a substrate for v-Src (Li *et al.*, 1996a,b), and has also been shown to copurify with c-Src in normal

cells (Lisanti *et al.*, 1994; Henke *et al.*, 1996; Li *et al.*, 1996a,b). Caveolin normally acts as a scaffolding protein to bind inactive signaling molecules, such as G α subunits, Ras, EGFR, and c-Src (Sargiacomo *et al.*, 1993; Lisanti *et al.*, 1994; Chang *et al.*, 1994; Li *et al.*, 1995; Couet *et al.*, 1997). It has also been shown that caveolin expression is down-modulated in cells transformed by various oncogenes (Koleske *et al.*, 1995), and that reexpression of caveolin in v-*abl*- and H-*ras*-transformed cells will abrogate anchorage-independent growth in these cell lines (Engelman *et al.*, 1997). Caveolin expression has also been shown by differential display and subtractive hybridization techniques to be down-regulated in human mammary carcinomas and several breast tumor cell lines compared with normal breast epithelium (Sager *et al.*, 1994). This evidence suggests that caveolin is inhibitory for transformation and that overexpression of c-Src may be deactivating caveolin through phosphorylation, leading to increased transformation.

b. Evidence for the HER1/c-Src Synergy Model in Human Breast Cancer

Simultaneous overexpression or activation of HER1 and c-Src in a significant portion of human breast tumors suggests that the two molecules might functionally interact in human tumors as they do in the 10T1/2 murine fibroblast model. This question was examined by Biscardi *et al.* (1998a,b), who analyzed a panel of 14 breast tumor cell lines and over 20 tissue samples for levels of HER1 and c-Src overexpression, association between c-Src and HER1, phosphorylation of Y845 and Y1101 on the receptor in complex with c-Src, increases in Shc phosphorylation, MAP kinase activation, and increases in tumor formation in nude mice. A direct correlation was found between the expression levels of c-Src and HER1 and the ability to detect stable interactions between the two kinases, the presence of the novel phosphorylations on the receptor, enhanced phosphorylation of downstream substrates, and tumor formation. Although not direct proof, results from these studies are consistent with those in the 10T1/2 model and suggest that c-Src and HER1 can functionally synergize to promote tumor progression when cooverexpressed in human tumors.

3. c-Src/HER2/*neu* INTERACTIONS

Because HER2/*neu* is so abundantly and frequently overexpressed in human tumors (particularly in breast cancers) and is oncogenic when overexpressed in cultured fibroblasts (DiFiore *et al.*, 1987b), an important question arises as to whether c-Src acts as a cotransducer to tumorigenic signals through HER2 as it does through HER1. Luttrell *et al.* (1994) showed that HER2 can be precipitated by the GSTc-SrcSH2 fusion protein from a hu-

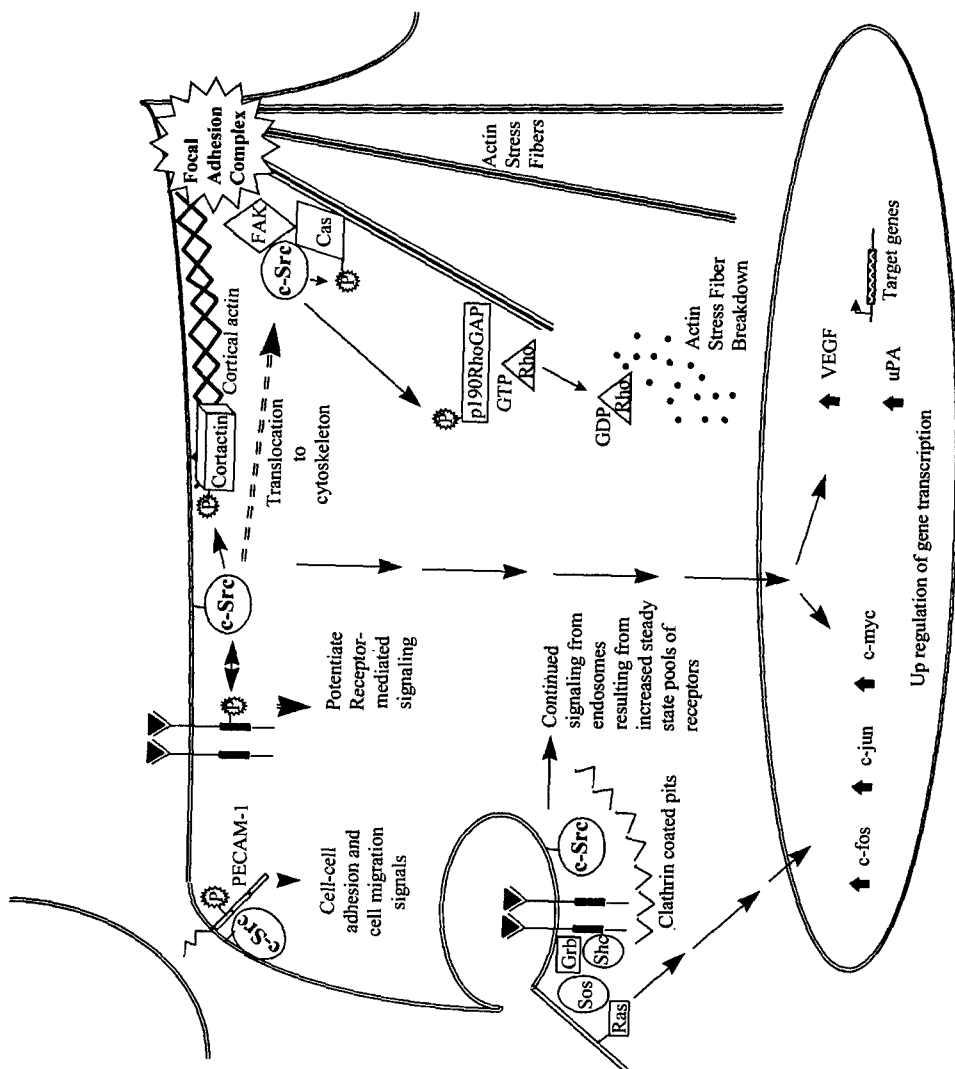
man breast cancer cell extract, suggesting that stable complexes may also form between c-Src and HER2 *in vivo*. c-Src association with and activation by HER2 was also shown in mammary tumors from HER2 transgenic mice (Muthuswamy *et al.*, 1994; Muthuswamy and Muller, 1995). Moreover, in coimmunoprecipitation studies our laboratory has detected c-Src in association with HER2 in 3 of 14 human breast tumor cell lines and in 3 of 13 tumor tissues (Belsches and Parsons, 1998). Cell lines exhibiting this complex respond to HRG mitogenically and tumorigenically, as measured by cell growth assays and colony formation in soft agar, in contrast to those cell lines that express HER2 but form no complex with c-Src. Interestingly, in contrast to the HER1, overexpression of neither HER2 nor c-Src is a prerequisite for detecting association between the two proteins. These data suggest that c-Src may potentiate HER2-dependent tumorigenicity through mechanisms similar as well as dissimilar to those described for HER1.

B. Targets of c-Src

1. TARGETS WHOSE EXPRESSION LEVELS ARE AFFECTED BY c-Src

The preceding discussion provides compelling evidence that RTKs can be direct targets of c-Src. Phosphorylation of specific sites by c-Src appears to regulate the shift from motility to mitogenesis in the case of the PDGFR (Hansen *et al.*, 1996) and the entrance into S phase of the cell cycle in the case of the EGFR (Fig. 3) (Tice *et al.*, 1998). Is c-Src capable of contributing to the malignant phenotype in ways other than through direct regulation of growth factor receptors? One alternative is the ability of c-Src to regulate gene transcription. Barone and Courtneidge (1995) showed that Myc was required to overcome a block of PDGF-induced DNA synthesis by kinase-deficient c-Src, suggesting that Src kinases control the transcriptional activation of Myc, which in turn can induce a program of gene transcription that

Fig. 3 Targets of c-Src and their potential roles in transformation. c-Src associates with and phosphorylates the ligand-activated EGF receptor, thereby potentiating downstream signaling from the receptor. This is manifested by increased levels of phosphorylated receptor substrates and augmented steady-state pools of internalized, activated receptors. In a reciprocal fashion, activated receptors can mediate activation and translocation of c-Src to the cytoskeleton, where it phosphorylates several substrates, including cortactin, p130CAS, and p190RhoGAP. These substrates are central to regulation of actin cytoskeleton rearrangements and thus signals that control morphological transformation and migration. c-Src also contributes to neoplastic development through cell-cell adhesion signaling and up-regulation of gene transcription. Similar types of interactions are thought to occur with other receptor tyrosine kinases known to associate with c-Src.



is required for growth. c-Src and v-Src have also been shown to up-regulate transcription of vascular endothelial growth factor (VEGF) (Rak *et al.*, 1995; Mukhopadhyay *et al.*, 1995a,b; Weissenberger *et al.*, 1997). VEGF is a multifunctional cytokine that alters the pattern of gene expression and stimulates the proliferation and migration of endothelial cells that line the walls of microcapillaries. VEGF treatment also renders these same cells hyperpermeable, thereby allowing plasma proteins access to the extracapillary space. This process, in turn, leads to profound alterations in the extracellular matrix that favor angiogenesis (reviewed in Klagsbrun and D'Amore, 1996). Another potent modulator of angiogenesis (reviewed in Tkachuk *et al.*, 1996) and metastasis (reviewed in Andreason *et al.*, 1997) is urokinase-type plasminogen activator (UPA), whose expression is up-regulated by v-Src (Bell *et al.*, 1990, 1993) and whose receptor is found in complex with c-Src family members (Bohuslav *et al.*, 1995). The ability of c-Src to influence gene transcription is a new and emerging question that is receiving considerable attention. However, most of the investigations that focus on the role of c-Src in neoplastic transformation have focused on substrates of c-Src and their contributions to development of the malignant phenotype. c-Src has a number of characterized substrates, most of which have functional connections to the actin cytoskeleton. These different substrates and their potential roles in transformation are discussed below.

2. TARGETS THAT SERVE AS SUBSTRATES OF c-Src

a. Focal Adhesion Kinase

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that localizes to focal adhesions and contributes to the processes of integrin-mediated cell spreading and migration through regulation of actin cytoskeleton remodeling (reviewed in Parsons and Parsons, 1997). FAK becomes tyrosine phosphorylated in response to various environmental stimuli, such as extracellular matrices and polypeptide and neuropeptide growth factors (reviewed in Schaller and Parsons, 1994). c-Src is intimately involved in FAK-mediated signaling. Activated c-Src (Y527F) is complexed with FAK through binding of the c-Src SH2 domain to Y397, the FAK autophosphorylation site (Schaller *et al.*, 1994; Cobb *et al.*, 1994). In addition, wt c-Src appears to be required for the FAK-mediated, integrin stimulation of mitogen-activated protein kinase (MAPK) (Schlaepfer and Hunter, 1997). Introduction of the amino-terminal half of c-Src, which lacks the kinase domain, reconstitutes integrin-induced MAPK activation in c-Src $-/-$ fibroblasts, thus the process appears to be independent of c-Src kinase activity (Schlaepfer *et al.*, 1997). This finding suggests a potential role for c-Src as a docking protein. The role of c-Src in cell spreading also appears to be independent of the kinase domain, because defects in spreading of fibroblasts derived from c-Src null mice can be restored by the SH2 and SH3 domains but not by the catalytic do-

main of c-Src (Kaplan *et al.*, 1994, 1995). These findings suggest that even though c-Src is activated in response to mitogenic factors such as EGF and HGF, it may require only the redistribution of c-Src to focal adhesions to stimulate motility and focal adhesion turnover.

Given that FAK is known to transduce signals involved in the regulation of cell adhesion and motility as well as the anchorage-independent growth of transformed cells, it would not be unexpected to find aberrant expression of FAK in human tumors. Indeed, increased expression or activation of FAK is observed in a number of human tumors, including sarcomas and carcinomas of the breast, prostate, and colon (Weiner *et al.*, 1994; Owens *et al.*, 1995; Withers *et al.*, 1996; Tremblay *et al.*, 1996). As might be expected with involvement of FAK in motility, the highest levels of FAK are seen in metastatic or invasive lesions (Weiner *et al.*, 1994; Owens *et al.*, 1995; Tremblay *et al.*, 1996). An increase in FAK phosphorylation also correlates with increased migration and invasiveness of squamous cell carcinoma cells treated with HGF (Matsumoto *et al.*, 1994). Together, the evidence suggests that signaling through FAK/c-Src complexes in normal and malignant cells is bidirectional. Integrins activate FAK, which can signal through c-Src to activate ERK2, or conversely, engaged growth factor receptors activate c-Src, which can then signal through FAK to mediate mitogenic or cytoskeletal responses.

b. p130CAS

p130Cas (CAS) was first identified as a highly tyrosine-phosphorylated protein in cells transformed by a variety of oncogenes (Mayer and Hanafusa, 1990; Auvinen *et al.*, 1995; Salgia *et al.*, 1996) and in normal cells following activation of integrins (Nojima *et al.*, 1995; Petch *et al.*, 1995; Vuori *et al.*, 1996) and stimulation with mitogenic neuropeptides, such as bombesin, vasopressin, and endothelin (Zachary *et al.*, 1992; Seufferlein and Rozen-gurt, 1994). The role of CAS in integrin or growth factor-mediated signaling is not understood. However, recent evidence indicates that CAS functions like an adapter molecule, binding a number of signaling molecules that participate in cell adhesion, such as FAK (Polte and Hanks, 1995; Harte *et al.*, 1996), PTP-PEST (Garton *et al.*, 1997), and Src family kinases (Polte and Hanks, 1995; Nakamoto *et al.*, 1996). Tyrosine phosphorylation of CAS is increased on adhesion and is largely dependent on c-Src (Vuori *et al.*, 1996; Hamasaki *et al.*, 1996). Thus, the link between cell adhesion, the actin cytoskeleton, and tumorigenesis is repeated, and the common involvement of c-Src in both processes suggests that c-Src may be a critical factor that links them together.

c. Cortactin

The v-Src and c-Src substrate, p75/p80/p85 cortactin, is an actin-binding protein that contains five tandem repeats in the N terminus and an SH3 domain in the extreme C terminus (Wu *et al.*, 1991; Maa *et al.*, 1992; Wu and

Parsons, 1993). The N terminus is responsible for the *in vitro* binding to actin. *In vivo* phosphoamino acid analysis of cortactin from primary chick embryo cells reveals that it contains only serine and threonine phosphorylations, whereas it exhibits constitutive phosphorylation on tyrosine in addition to serine and threonine in cells transformed by activated c-Src (Y527F) (Wu *et al.*, 1991). In immortalized murine 10T1/2 fibroblasts, cortactin has a low basal level of tyrosine phosphorylation that is increased on both EGF stimulation and c-Src overexpression (Wilson and Parsons, 1990; Maa *et al.*, 1992). Cortactin has also been shown to be tyrosine phosphorylated in response to fibroblast growth factors (Zhan *et al.*, 1993, 1994). These observations suggest that cortactin may be a substrate of growth factor receptors as well as of c-Src or that c-Src mediates growth factor-induced tyrosine phosphorylation of cortactin. The findings that cortactin associates with the Src SH2 domain and colocalizes with v-Src in transformed cells (Okamura and Resh, 1995), and that increased tyrosine phosphorylation of cortactin is seen in CSK-deficient cells, favor the notion that c-Src and/or its family members are responsible for phosphorylating cortactin (Nada *et al.*, 1994; Thomas *et al.*, 1995). Interestingly, two phases of EGF-induced cortactin tyrosine phosphorylation can be observed in 10T1/2 cells, one occurring within 2–10 min following stimulation and another occurring later in G₁, with the maximum level seen approximately 9 hr posttreatment. In both cases, the level of phosphorylation is increased by overexpression of c-Src (Maa *et al.*, 1992). These observations raise the question of whether cortactin may function in mid-late G₁ as well as in immediate-early G₁.

Indirect immunofluorescence microscopy of 10T1/2 cells reveals that cortactin is localized within the cytoplasm to punctate sites that are concentrated around the nucleus and colocalized with actin at the plasma membrane and peripheral adhesion site (Maa *et al.*, 1992). This pattern is not altered on EGF treatment or c-Src overexpression in 10T1/2 cells. However, in v-Src- or Y527F-c-Src-transformed cells, cortactin is localized to modified focal adhesions, termed podosomes. The appearance of podosomes is associated with loss of adhesive properties (Marchisio *et al.*, 1987; Wu *et al.*, 1991). Cortactin is also localized to podosome-like cell matrix sites in human tumors that overexpress the protein (including carcinomas of the breast and head and neck) (Schuuring *et al.*, 1992; Brookes *et al.*, 1993; Williams *et al.*, 1993; Schuuring *et al.*, 1993; Meredith *et al.*, 1995; Campbell *et al.*, 1996; van Damme *et al.*, 1997). From these observations, it follows that abnormal subcellular distribution of cortactin in human carcinomas may play a role in deregulating important protein–protein interactions that may be required for the proper formation of cell matrix contact sites. In support of this hypothesis is the correlation of cortactin overexpression with increased invasiveness, metastasis, and a poorer patient prognosis (Williams *et al.*, 1993; Meredith *et al.*, 1995; Takes *et al.*, 1997).

d. p190RhoGAP

p190RhoGAP was first identified as a tyrosine-phosphorylated protein that coprecipitates with p120RasGAP from v-Src-transformed Rat-2 cells (Ellis *et al.*, 1990). p190 has an N-terminal domain that binds GTP (Settleman *et al.*, 1992b; Foster *et al.*, 1994) and a C-terminal GTPase-activating domain (GAP) that is specific for small GTP-binding proteins of the Rho family (Settleman *et al.*, 1992a). p190 is functionally linked to the actin cytoskeleton through its ability to stimulate the conversion of Rho-GTP (which stimulates stress fiber formation) to Rho-GDP (which permits actin disassembly) (Ridley and Hall, 1992). Two phosphorylation sites, Y1087 and Y1105, in the middle portion of the molecule are postulated to mediate binding to the two SH2 domains of p120RasGAP (Bryant *et al.*, 1995; Hu and Settleman, 1997), although of the two sites, only Y1105 phosphorylation can be detected *in vivo*. Overexpression of c-Src in 10T1/2 cells results in an increase in the basal tyrosine phosphorylation of p190, specifically at Y1105 (Roof *et al.*, 1998). This evidence, along with the findings that overexpression of kinase-deficient c-Src decreases the phosphorylation at Y1105 and that c-Src phosphorylates Y1105 *in vitro*, suggests that c-Src is directly responsible for phosphorylation of this residue. Levels of p190 tyrosine phosphorylation are generally correlated with levels of the p190/p120RasGAP complex that can be detected *in vivo*, suggesting that high levels of p190 tyrosine phosphorylation could bind more RasGAP, thereby sequestering RasGAP away from Ras and permitting Ras to remain in the active, GTP-bound state longer. This scenario is consistent with the role of c-Src as a comitogenic signaling partner of growth factor receptors.

Although EGF treatment of 10T1/2 cells does not cause a further increase in tyrosine phosphorylation of p190, it does cause a rapid (seconds to minutes) and transient redistribution of p190 from a diffuse cytoplasmic localization into concentric arcs that radiate away from the nucleus with a time course that mimics EGF-stimulated actin dissolution (Chang *et al.*, 1995). Overexpression of wt c-Src expands the window of time in which EGF-induced actin dissolution and p190 arc formation occur, whereas overexpression of kinase-deficient c-Src contract the window. These results correlate with the level of p190 tyrosine phosphorylation and implicate another role for c-Src in regulating cytoskeletal reorganization, possibly by inactivating Rho through activation and redistribution of p190RhoGAP.

e. Platelet Endothelial Cell Adhesion Molecule

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a 130-kDa glycoprotein of the immunoglobulin gene superfamily that localizes to points of contact between confluent endothelial cells (Newman *et al.*, 1990; Tanaka *et al.*, 1992). On induction of endothelial sheet migration, PECAM-1 becomes diffusely organized within the cytoplasm, and ectopic expression of

the gene inhibits cell migration, suggesting that translocation from the periphery to the cytoplasm is a mechanism by which the inhibitory action of PECAM-1 is relieved (Schimmenti *et al.*, 1992). The cytoplasmic tail of PECAM-1 is critical for cell surface activity (DeLisser *et al.*, 1994; Yan *et al.*, 1995). It contains immunoreceptor tyrosine-based activation motifs (ITAMS) that are phosphorylated by c-Src *in vitro* and *in vivo* and bind c-Src SH2 domains *in vitro* (Lu *et al.*, 1997). Several lines of evidence suggest that tyrosine phosphorylation is involved in transducing cell migration signals through this molecule (Lu *et al.*, 1996; Pinter *et al.*, 1997). Tyrosines 663 and 686 appear to be the major sites of tyrosine phosphorylation, because mutation of either residue results in a drastic reduction in tyrosine phosphorylation, and mutation of Y686 is associated with a reversal of the PECAM-1-mediated inhibition of cell migration (Lu *et al.*, 1996). Again, phosphorylation by c-Src is a potential mechanism of regulation of a molecule involved in cell-cell contacts and migration, pointing to a role for both PECAM-1 and c-Src in angiogenesis and metastasis.

f. Other Substrates

Additional reports implicate still other c-Src substrates in cell-cell adhesion. Syndecan-1 is a cell surface proteoglycan that interacts with extracellular matrix molecules and growth factors to maintain epithelial cell morphology, anchorage-dependent growth, and inhibition of invasiveness in cell culture assays. The absence of this molecule correlates with a higher grade of transformation and poorer patient prognosis (Inki and Jalkanen, 1996). Its expression is negatively regulated at the level of translation on transformation by polyoma virus middle-T antigen. The effects of middle-T antigen are dependent on association with and activation of c-Src (Levy *et al.*, 1996). c-Src may also function in processes other than those related to cytoskeletal or adhesion dynamics but that still lead or contribute to a transformed phenotype. For example, a role for c-Src in mitosis has been implicated through the identification of a 68-kDa RNA-binding protein, called Sam68 (Src-associated in mitosis), that binds the SH3/2 domains of c-Src (reviewed in Courtneidge and Fumagali, 1994) and is postulated to act through c-Src to regulate microtubule dynamics via association with (Abu-Amer *et al.*, 1997) and phosphorylation of (Matten *et al.*, 1990) tubulin.

V. POTENTIAL THERAPEUTIC APPLICATIONS OF c-Src/HER1 INTERACTIONS

Along with the first evidence for possible roles for receptor and nonreceptor tyrosine kinases in human tumorigenesis has come the development of strategies to inhibit the functions of these classes of enzymes. A plethora of

inhibitors based on various structural and functional characteristics of the enzymes have been developed. First and foremost among these are inhibitors of catalytic activity. Among these inhibitors are the tyrphostins (Burke, 1992; Levitzki and Gazit, 1995), which compete with the protein substrate for access to the catalytic site; genistein (Akiyama *et al.*, 1987), a competitive inhibitor of ATP; lavendustins A and B (Onoda *et al.*, 1989); erbstatin (Imoto *et al.*, 1987); and herbimycin A (Uehara *et al.*, 1986, 1989a,b), which has been shown to promote the ubiquitin-based degradation of the TKs (Sepp-Lorenzino *et al.*, 1995). Other inhibitors are designed to prevent interactions mediated through SH2 and SH3 domains (peptidomimetics) (Smithgall, 1995; Plummer *et al.*, 1996, 1997) or to prevent myristylation (*N*-fatty acyl glycinol compounds) (Shoji *et al.*, 1990). In numerous cases these reagents have been demonstrated to be antiproliferative (Clark *et al.*, 1996; Traxler *et al.*, 1997; Hartmann *et al.*, 1997). In other instances membrane penetrance of the drug has been a problem in testing their efficacies in tissue culture and animal models (Gilmer *et al.*, 1994).

Studies characterizing the molecular interactions between c-Src and HER1 have revealed an additional target for drug design, specifically the sequences surrounding Y845 of HER1. Phosphorylation of this site by c-Src appears to be required for the mitogenic and tumorigenic aspect of receptor function, as shown by the inability of kinase-defective c-Src to phosphorylate Y845 and the nonfunctionality of the mutant Y845F receptor. In human tumors that overexpress c-Src and HER1, inhibiting the ability of c-Src to phosphorylate Y845 might reduce the tumorigenic potential of the overexpressed receptor as well as the ability of c-Src to synergize with the receptor. Such inhibition might be accomplished by a Y845 peptidomimetic. The advantages appear to be that this inhibition targets an enzyme/substrate interaction that occurs to the greatest degree in those cells that overexpress both players (c-Src and HER1, respectively), namely, cancer cells. In no normal cells are these two molecules known to be simultaneously overexpressed. For example, in the adult, the highest levels of c-Src are found in platelets (Golden *et al.*, 1986) and in cells of the nervous system, whereas high levels of HER1 are found in the liver and kidney (Nexo and Kryger-Baggensen, 1989). In theory, therefore, the Y845 peptidomimetic might be more likely to target the tumor cells than the normal cells, thus providing a potential "tumor-specific" drug for cancers such as carcinomas of the colon, breast, and lung.

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